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**13. ABSTRACT** *(Maximum 200* Two conditions of Schwann cell hyperplasia occur at an increased rate of incidence in NF1 patients: neurofibromas and malignant Schwannomas. From the funded research, the expression of stem cell factor (SCF) isoforms and of Kit, the SCF receptor, in neurofibroma lesions and in 4 malignant Schwannoma lines was determined. Each of the four human malignant schwannoma lines tested using an RNase protection assay (RPA) expresses the exon 6-lacking (membrane-associated) isoform of SCF exclusively, while the majority of human fibroblast sources express mostly the soluble isoform. Analysis of RNA extracted from neurofibroma tissue revealed expression of primarily the soluble isoform of SCF by this mixed population of cells. ELISA from conditioned media from the cell lines used confirmed the RPA data. Kit expression by malignant Schwannoma lines was indicated by immunofluorescent staining with subsequent analysis by confocal fluorescent imaging, immunoprecipitation, and western blotting. Unlike malignant schwannomas, hyperplastic Schwann cells from NF1 related and non-NF1 related neurofibromas do not express Kit protein. A tetracycline-inducible anti-Kit ribozyme system was developed to inhibit Kit expression. Overall, the results suggest that SCF isoform expression is tightly regulated in Schwann cells and that any Kit expression in Schwann cells may be related to a transformed phenotype.

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Thomas F. Huff 10-16-96

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**Introduction**:

Type 1 neurofibromatosis (NF1) is among the most common inherited human diseases, affecting one out of 3500 individuals worldwide (1). The responsible gene has been cloned and is located on the long arm of chromosome 17 (2). The NF1 cDNA reveals that the gene encodes a large protein, called neurofibromin, which contains a region with homology to mammalian Ras GTPase-activating protein (GAP) (3). It has been reported that this region indeed has GAP-like activity against *ras* p21, supporting the postulation that neurofibromin functions as a tumor suppressor (4). Two conditions of Schwann cell hyperplasia occur at an increased rate of incidence in NF1 patients: neurofibromas and malignant schwannomas. Neurofibromas, a hallmark characteristic of the disease, are benign peripheral nerve tumors consisting primarily of Schwann cells, which account for 60-85% of the cellular composition. Fibroblasts make up an additional 10-20%, with pericytes, perineurial cells, mast cells, endothelial cells and smooth muscle cells also present (5). It has long been known that neurofibroma tissues contain increased numbers of mast cells (6, 7), but the mechanism by which this occurs has not been well understood. Malignant schwannomas occur much less frequently, with an incidence of up to 2% in NF1 patients (8, 19). This is, however a significantly higher rate of occurrence than that seen in genetically normal individuals (<<1% (9)). Although it seems plausible that the NF1 gene defect alone could be responsible for these hyperplasias, this does not appear to be the case. All patients with NF1 appear to have mutations within the same region of chromosome 17 (2, 10-11), but not all have neurofibromas (8, 12) and even fewer develop malignant schwannomas, suggesting that additional mutations or other influences are required (13).

Our laboratory first provided evidence of a soluble factor produced by murine fibroblasts which can promote the growth of mast cells in vitro (14). Since then the factor, now known as stem cell factor (SCF), has been more fully characterized in both rodent and human, and is known to exist as either a soluble or membrane-bound protein, dependent upon whether the portion of the primary gene transcript encoded by exon 6 is included in the final messenger RNA (15). The exon 6-containing mRNA gives rise to a protein that contains a protease-sensitive site in an extracellular membrane-proximal location. This isoform of SCF, after being initially expressed as a membrane-inserted protein, is efficiently cleaved by an unidentified protease, releasing a biologically active growth factor. Soluble SCF has been described as a potent chemotactic agent for cells that express its receptor Kit, the product of the protooncogene c-*kit* (16, 20). We recently reported that normal rodent and human Schwann cells, and the human malignant schwannoma cell line ST88-14, all produce SCF (17). Interestingly, Hirota, et al. noted higher levels of stem cell factor mRNA in neurofibroma tissue compared with normal skin and suggested that SCF may be involved in the increased numbers of mast cells in these lesions (18) Since mast cells express the Kit receptor, one might speculate that the mast cells in a neurofibroma may be recruited to the site by the chemotactic activity of soluble SCF. We have also reported that, although normal rodent and human Schwann cells do not express Kit, the ST88-14 malignant schwannoma line expresses Kit mRNA and protein (17). These findings have led us to consider whether the Schwann cell hyperplasia found in a neurofibroma might be due to the simultaneous expression of SCF and its receptor by the resident Schwann cells, resulting in an autocrine growth loop. In this study, we evaluate Kit expression in three additional human malignant schwannoma cell lines and in excised neurofibroma and schwannoma tumors. In addition, we determine which isoform of SCF mRNA is produced by these and other sources.

In the original grant application, we proposed to address the following three questions:

Objective 1: Is SCF, which is produced in Schwann cell lines, also produced in normal Schwann cells or Schwann cells from NF lesions ? What is the pattern of isoform expression of the soluble or membrane-inserted stem cell factor?

Objective 2: Is c-*kit*, which is aberrantly expressed in a human schwannoma line but not in normal Schwann cell cultures and lines, also aberrantly expressed on freshly isolated NF-derived Schwann cells but not normal Schwann cells ?

Objective 3: Do mast cells produce SCF-releasable cytokines which potentiate the growth of Schwann cells from normal tissue or from NF lesions ?

At the time of the 15 month progress report, we updated our objectives to take advantage of several experimental findings we had made. These updates improved each of the three objectives stated in the original application:

1. In addition to using quantitative PCR to determine relative levels of SCF isoform expression (Objective 1 in the original application), we proposed to use RNAse protection to gather the same information. Because we can also address our questions in cell lines, we should have enough RNA to do RNAse protection. For primary isolates of tissue, we still intend to use quantitative PCR with RNA control fragments.

2. The availability of the human mast cell line HMC-1 greatly expanded our possibilities for addressing the effects of mast cells on Schwann cells and vice versa (Objective 3 in the original application). We proposed to assess HMC-1 cells after coculture with Schwann cells for phenotypic changes.

3. Two new growth factor/receptor complexes (*Neu* differentiation factor and Flk-2 ligand), in addition to SCF/Kit, were proposed to be examined for their possible role in mast cell-Schwann cell modulation.

4. A powerful new possibility for addressing the possibility for a Kit autocrine loop in malignant schwannomas was proposed, specifically to use an tetracycline-inducible antisense ribozyme to Kit. This strategy was the basis for an AASERT supplement grant for Mr. Christopher Shelburne, a graduate student in the lab. The AASERT was subsequently funded, and details of Mr. Shelburne's work are included in this final report.

   As an introduction to the work funded by the supplement to this grant, Kit is a 150Kd Receptor Tyrosine Kinase whose expression is required for normal mast cell development, and, as we reported, is aberrantly expressed in the ST88-14 malignant schwannoma line. Previous studies in our laboratory have used antisense technology on in-*vivo* derived mast cell populations to address the role Kit played in mast cell maturations and proliferation. In the supplement application, we proposed to utilize these techniques first on murine mast cell lines and then in the human malignant schwannoma line ST88-14 in order to address the possibility of a Kit-SCF autocrine loop in these malignant schwannomas which show increased incidence in NF-1 patients.. However, conventional antisense techniques proved to be unfeasible. Therefore, we decided to approach the problem of inhibiting Kit protein expression by using an intracellular expression system. Two challenges were directly

responsible for the approach we decided to take. First, was the realization that many mast cell lines (including P815) contain activating mutations in the Kit protein which render the cell line dependent upon its expression. This requirement for Kit expression demanded that our expression system be inducible. Second, the high levels of c-*kit* expression in many of these lines required that we use an aggressive and efficient methodology for inhibiting c-*kit* expression.

In order to accomplish the second objective, we decided to use a novel approach featuring an anti c-*kit* hammerhead ribozyme. Hammerhead ribozymes are a group of small self cleaving RNAs first described as the replicative cycle in plant viroids. Hammerhead ribozymes are capable of mediating the cleavage of a phosphodiester backbone through a cation dependent $SN_2$ reaction. Cleavage occurs at a GTX triplet, where X is usually a cytosine. Cleavage is mediated by hybridization of sequences flanking the catalytic core of the ribozyme to the flanking regions surrounding the GTX triplet. Adaptation of the catalytic core of the hammerhead ribozyme to function as a tool for cleaving non-self RNAs has been successfully used to target numerous protein encoding RNAs. The advantage of a ribozyme over conventional antisense techniques is that one ribozyme may cleave numerous targets. This study was conducted in order to develop and successfully express an inducibly regulated anti-c-*kit* ribozyme first in the murine mastocytoma P815 and then in the human malignant schwannoma line ST88-14.

**Body**:

*The body of the final report will follow a similar format as that used in the 15 month report, i.e. an alphabetic listing of findings with reference to Figures in the Appendix. The findings obtained from the AASERT supplement are included beginning in the N subsection. To emphasize the major findings conceptually, all standard procedures have been described in a Materials and General Procedures subsection at the end of this section.*

A. The preliminary data cited in the original grant application has been completed and published concerning the ST88-14 schwannoma line (6). Additional confirmatory experiments were performed as part of the funded research to address reviewers concerns. Reprints are available upon request; however a summary of the results is excerpted below because progress report instruction state "journal articles may not be used in toto as any part of the report":

> Schwann cells are primary cell type in the disfiguring lesions associated with neurofibromatosis type 1 (NF-1). These lesions also contain abnormally high numbers of mast cells, a cell type which develops in response to stem cell factor. We report here that neonatal and adult rat and human Schwann cells, as well as a transfected rat Schwann cell line and a human schwannoma line derived from an NF-1 patient, all produced stem cell factor mRNA and protein. In coculture experiments, surface expression of stem cell factor by neonatal rat Schwann cells was profoundly downregulated by contact with dorsal root ganglia neurites. The receptor for stem cell factor, Kit was not expressed in normal Schwann cells but was expressed in the human schwannoma line, suggesting that aberrant Kit expression may form an autocrine loop in certain Schwann cell neoplasias.

B. Two internal control fragment for RT-PCR of mouse stem cell factor have been constructed.

In the first strategy, we have added nucleotides to the native mRNA sequence and in the second, we have deleted nucleotides. As described in the original grant application, the difference in molecular weight allows us to visualize different bands on gels.

In the first strategy, a 102 bp fragment was cut from the polylinker site of pBluescript II SK+ by digestion with KpnI and SstI. The fragment was gel-purified and ligated into a unique NsiI site within the SCF sequence of our mouse SCF clone. By *in vitro* transcription, an RNA transcript of the plasmid was generated which is 102 bp larger than the naturally occurring mRNA species. The resulting ribocontrol fragment were to be used, at varying concentrations, in competitive RT-PCR reactions with RNA isolated from the cells or tissue being studied. However, we consistently encountered problems with the PCR amplification of this fragment (Figure 1). We believe that this is likely due to our use of a repeated sequence from the Bluescript plasmid as our insert sequence. Therefore, we constructed a second RNA control fragment, this one being smaller than the target RNA, by the strategy outlined in the paragraph below.

The human SCF clone was first digested with the restriction endonuclease AatII. The SCF sequence contains a unique recognition site for this enzyme (cleavage occurs between nucleotides 677 and 678 by our numbering). The DNA was phenol-chloroform extracted and ethanol precipitated. A second restriction digest was performed, using the enzyme MunI. Digestion with this enzyme results in an additional unique cleavage between nucleotides 784 and 785. Another phenol-chloroform extraction and ethanol precipitation was performed. The resulting overhanging ends were blunt-ended with T4 DNA polymerase. This enzyme exhibits 3' to 5' exonuclease activity (which removes the 3' overhang left by AatII digestion) as well as 5' to 3' polymerase activity (which fills in the recessed 3' end left by MunI digestion). The DNA was phenol-chloroform extracted and ethanol precipitated, followed by drop dialysis for four hours. The blunt-ended, drop-dialyzed plasmid (minus 111 base pairs between AatII and MunI sites) was religated with T4 DNA ligase. *E. coli* HB101 bacteria will be transformed with the smaller plasmid for amplification. This plasmid can be used to generate RNA transcripts which will be used in competitive RT-PCR reactions with total RNA extracted from the cells or tissue under study.

C. PCR primers have been designed such that the soluble (KL-1) isoform can be specifically amplified. This was accomplished by constructing a 3' primer which is complementary to the exon 6-encoded sequence in the mRNA. A second 3' primer, which targets a region upstream of the exon 6 sequence, is used to amplify all SCF mRNAs without discriminating between isoforms.
    1. By doing competitive RT-PCR with each set of primers, the relative levels of expression of KL-1 mRNA and total SCF mRNA can be determined. From these data, a KL-1:KL-2 ratio can be calculated.
    2. After an early failure using one primer set (Figure 2), a second primer set has now been obtained for both the mouse and human systems (Figure 3). A later set of experiments allowed the first primer set to work at a lower annealing temperature (Figure 4). The concentration of work at this time is in the human system.

D. Four human malignant schwannoma cell lines (ST88-14, NF-1T, STS-26T, and T26-2C) have been obtained, grown to determine optimal tissue conditions for RNA, and mRNA has been isolated. Preliminary RT-PCR experiments have detected mRNA for stem cell factor in all four cell lines. c-*kit* mRNA has been detected in ST88-14 cells. The presence or absence of c-*kit* mRNA was confirmed in the other three cell lines as documented later in this final report.

E. A human mast cell line, HMC-1, has been obtained for use in co-cultures with human schwannoma lines. The HMC-1 cell line has been described (7,8) as having characteristics of an immature mast cell line. We have speculated that, in the lesions of NF1, mast cell precursors may be recruited to the site (perhaps by SCF produced by resident Schwann cells), at which time they are induced to proliferate, mature, and perhaps release cytokines which, in turn, induce the Schwann cells to proliferate. In light of this hypothesis, we have incubated HMC-1 cultures for four hours in the presence of 1 μM ionomycin, after which total RNA was extracted. RT-PCR experiments will be conducted to compare mRNA expression by unstimulated vs. stimulated HMC-1 cells. We will assess expression of mRNA for cytokines such as NDF, *flk*-2 ligand (see G below), as well as for other markers of mature mast cells (9,10), such as the alpha chain of the high affinity receptor for IgE (FcεRIα), and the mast cell-specific proteases tryptase and chymase. In addition to ionomycin stimulation, we will also determine whether the HMC-1 cells are driven to a more mature phenotype upon coculture with the schwannoma cell lines.

Also, two new mouse mast cell lines have been generated for use in co-cultures with the SV-40 transformed rat Schwann cell line. These lines were generated by a graduate student, Chris Shelburne, and we have used another project based on his mast cell expertise as the basis for application a supplement to this grant. The mouse mast cell lines are strongly Kit positive but, like HMC-1 human mast cells, have an immature granule phenotype.

F. An RNAse protection fragment capable of discriminating between the SCF isoforms has been constructed. PCR primers were designed to amplify a 222 bp fragment of the human SCF sequence:

Primer 1: 5' - ATG **GGA TCC** ATT CAA GAG CCC AGA AC - 3'

The nucleotides printed in bold type comprise a recognition site for the restriction endonuclease BamHI. The underlined nucleotides have specificity for sequence near the 5' end of exon 5 of the hSCF cDNA (nucleotides 936-952 by the our numbering).

Primer 2: 5' - GC**T CTA GA**T GCT ACT GCT GTC ATT CC - 3'

The bold type nucleotides represent a recognition site for cleavage by XbaI. The underlined sequence is specific for a region near the 3' end of hSCF exon 6 (nucleotides 1156-1139).

30 cycles of PCR were performed using 0.2μg of each primer. 10ng of plasmid DNA containing the hSCF clone were used as template. Ten separate 75μl reactions were carried out, and the products were combined into one tube. The DNA was phenol-chloroform extracted and ethanol precipitated. Restriction digests were performed on the PCR product and, in a separate reaction, on pBluescript II SK+, using both BamHI and XbaI. After

digestion, the DNA was again phenol- chloroform extracted and ethanol precipitated. Both of the samples were drop-dialyzed for four hours prior to ligation. Three ligation reactions were performed using different ratios of double-digested vector and double-digested insert.

The products were used to transform competent *E. coli* HB101 bacteria. Successful cloning of the fragment was confirmed by digestion of plasmid DNA obtained from the transformed bacteria with BssHII. This enzyme cuts at two sites which flank the multiple cloning site of pBluescript II SK+. Digestion of plasmids containing no insert releases a fragment of 173 base pairs. If the insert is present, the released fragment is 388 base pairs in size.

G. Schwann cell lines and the HMC-1 human mast cell line have been co-cultured in preliminary experiments to determine relative radiosensitivities. Plastic vials containing 1X $10^6$ cells in one ml of medium were placed in a Mark I irradiator (J.L. Shepherd and Associates) which houses a $^{137}$Cesium source. Six sets of vials were subjected radiation intensities of 0, 500, 1000, 2000, 3000, and 4000 rads, respectively. The cells were then cultured in 25 $cm^2$ flasks containing 5 ml of media.

The cultures are being examined daily to determine at which radiation intensity the proliferation of the cells can be halted without resulting in cell death. Preliminary observations suggest that 3000 rads will prove to be the optimal intensity. When this is confirmed, sufficient cell numbers will be irradiated such that they will form an adherent monolayer which will remain viable, but which will not proliferate. HMC-1 cells will be added to these monolayers and cultured for various time periods, after which phenotypic characterizations will be made and In order to determine what effect mast cells have on Schwann cells, mast cell lines are irradiated. In order to determine what effects Schwann cells have on mast cell growth, Schwann cell lines will be irradiated.

H. All Schwann cell lines and the HMC-1 human mast cell have been characterized for expression of mRNA not only for Kit and SCF, but also for two receptor/ligand complexes: Flk-2 receptor tyrosine kinase (11,12) and its ligand; and HER2/*neu* and its ligand *neu* differentiation factor (NDF) (13). RT-PCR was performed using primers specific for human *neu* differentiation factor, the ligand for the HER2/*neu* receptor. Total RNA was extracted from HMC-1 cells, each of the four human malignant schwannoma cell lines, the human fibrosarcoma cell line HT1080, and the human fibroblast cell line MRC-9. 1 μg of each RNA sample was reverse transcribed into cDNA, using an antisense primer specific for a sequence near the 3' region of the NDF mRNA. The resulting cDNAs were PCR-amplified, using the above primer as well as a sense primer with similar specificity for NDF sequence.

Following amplification, the products were resolved by agarose gel electrophoresis, and subjected to Southern blot and autoradiographic analysis. The expected 875 bp product was detected in the HT1080, MRC-9, ST88-14, and STS-26T cell lines. A faster migrating band was detected in the T265-2c sample. This smaller product may represent a different isoform of NDF.

It is interesting that mRNA for NDF was detected in some of the schwannoma cell lines, in that Schwann cells have been reported to express the HER2/neu receptor. Indeed, Schwann cells derived from primary cultures of surgical specimens of nerve tissue may specifically induced to proliferate upon addition of recombinant NDF to the culture medium. NDF may also be expressed by normal human Schwann cells, or its expression by some malignant schwannoma cell lines may be ectopic in nature. It is possible that ectopic expression of this factor may contribute to the transformed phenotype of these cells.

I. A dominant negative construct of the c-*kit* gene product was obtained from Dr. Geoffrey Krystal using a partial length human c-*kit* clone which we had generated ligated to another c-kit clone with additional 3' sequence. Dr. Krystal mutated the resulting full-length clone such that the kinase cassette was interrupted and thus cannot signal. This transfectable construct has been successfully used by Dr. Krystal to test for the possibility of autocrine loops in lines derived from acute myeloblastic leukemia, from small cell lung carcinoma, and from breast cancer. The transfected defective Kit competes with wild type Kit. This was placed in the pCEP 4 vector for using in transfection experiments. More importantly, Dr. Krystal used this colony to partially delete the kinase cassette such that the Kit is unable to transduce a signal. This dominant negative construct has been used by Dr. Krystal to test for the possibility of Kit/SCF autocrine loops in breast cancer, small cell lung cancer and acute myeloblastic leukemia. The transfected defective Kit competes with wild type Kit for the endogenously produced SCF and, thus, inhibits Kit signaling. We have established optimal transfection conditions for the four schwannoma lines and have done preliminary transfections with both of Dr. Krystal's constructs.

J. RNAse protection assay for SCF isoforms.

In order to determine the relative levels of expression of the two SCF messenger RNA isoforms, an RNase protection assay (RPA) was performed using a probe which is complementary to the sequence including most of exons 5 and 6. A uniformly radiolabeled RNA transcript containing a sequence complementary to most of exons 5 and 6 of the human stem cell factor mRNA was generated by *in vitro* transcription and allowed to hybridize with 10μg of total RNA. Single stranded RNA was digested with a mixture of RNases A and $T_1$, and the products were resolved by polyacrylamide gel electrophoresis. The presence or absence of exon 6 is indicated by a protected fragment of 222 or 144 nucleotides, respectively. (Figure 6). A protected fragment 222 nucleotides in length results from hybridization of the probe with an mRNA which includes sequence encoded by exon 6 (the soluble isoform). When exon 6 is absent, as in the case of the membrane-bound isoform, the protected fragment is 144 nucleotides long. Figure 7 is representative of several RPA experiments. In each of four human malignant schwannoma cell lines tested, a protected fragment of an apparent length of 144 nucleotides results, whereas no 222 nucleotide fragments are evident. This suggests that these cell lines exclusively express mRNA for the membrane-bound isoform of SCF. In contrast to these data, RNA extracted from a neurofibroma lesion yields a protected fragment of a size consistent with the soluble isoform only. The human fibroblast cell lines CCD18, HS68, and MRC-9 also expressed only the soluble isoform, as did the human fibrosarcoma cell line HT1080 (Figure 8). In addition, RNA extracted from fibroblasts cultured from explanted normal human skin and keloid tissue all expressed only the mRNA for soluble SCF (Figure 9).

K. ELISA for soluble SCF in cell-free supernatants:

To evaluate whether expression of the soluble isoform of SCF mRNA, as indicated by RPA, was consistent with release of soluble SCF into the culture media, we performed ELISA analyses on the conditioned media of the cell lines used (Figure 10). No SCF protein was detected in the conditioned media of any of the four malignant schwannoma cell lines, consistent with the RPA results. However, SCF was detectable in the conditioned media of all the cell lines shown to express mRNA for the soluble isoform.

L. Immunohistochemistry for Kit expression.

Thin sections (7μm) of formalin-fixed paraffin-embedded neurofibromas were evaluated for the expression of Kit protein by immunohistochemistry as described in materials and methods. Neurofibromas are composed primarily of Schwann cells, but include a few other cell types, including mast cells. We wished to determine whether these Schwann cells express the Kit receptor, offering a possible explanation for their hyperplasia. Mast cells are known to express Kit protein, and therefore serve as convenient internal controls for the staining protocol. Indeed, Kit protein was easily detectable on mast cells (Figure 11, right panel). No expression is seen in the isotype matched control (left panel). The surrounding Schwann cells, however, show no evidence of immunoreactive protein.

M. RNase assay for Kit expression

RPA was performed using a probe containing nucleotides 1-374 of the 5' end of the c-kit cDNA. 10 μg of total RNA from each of the four malignant schwannoma lines were used for hybridization. HMC-1 RNA was used as a positive control. The result was that a protected fragment of a size consistent with 374 nucleotides was obtained with the HMC-1 sample, but no protected fragments resulted from any of the schwannoma lines.

## Subsection on anti-Kit ribozyme

**(This subsection represents work done as part of a funded graduate student supplement to this grant.)**

The possibility that was investigated in this subsection was that an autocrine growth loop between stem cell factor and aberrantly-expressed c-kit, its receptor, contributes to the transformed state in these malignant schwannomas. The model system that Mr. Shelburne developed was a new and more powerful way to address this possibility using the malignant schwannoma lines we already have in hand. This approach utilized inhibition of *c-kit* expression by an inducible anti-*c-kit* mRNA hammerhead ribozyme. Ribozyme activity has been shown to be highly specific and efficient in destroying target mRNA both *in-vitro* and in-vivo. The hammerhead ribozyme coding sequence (Figure 12) was shown to function in all *in-vitro* translation experiments. This ribozyme has been designed to target the base triplet GTC (25), located at position 1609 in the *c-kit* mRNA, as determined by the cDNA for murine *c-kit* (26). The ribozyme structure is flanked by two 7 bp

regions which will hybridize to the flanking sequences of the target GTC region in the *c-kit* mRNA. Hybridization of these sequences in the presence of a divalent cation such as $Mg^{2+}$, or $Mn^{2+}$, will drive the formation of the hammerhead structure. Cleavage of the mRNA then proceeds with a straightforward $SN_2$ nucleophilic attack initiated by the catalytic core of the ribozyme (27). Cleavage of the mRNA occurs at the cytosine.

Ribozyme expression was controlled using a tetracycline inducible promoter system (Figure 13). This system (28) consists of two plasmids, pUDHD10-3 and pUDH15-1. 15-1 encodes a fusion protein (tTA) which consists of the E.Coli TN10 tetracycline resistance protein and the carboxy terminus of the VP16 viral protein. This protein binds a promoter sequence in 10-3, which consists of multiple repressor binding domains and the CMV promoter. This sequence lies upstream of a Multi-Cloning Site into which the ribozyme coding strand will be cloned. In the presence of tetracycline, the fusion protein will be prevented from binding the promoter, and transcription of the ribozyme will be prevented. In the absence of tetracycline, the tTA protein will bind the promoter and activate transcription. This promoter system has distinct advantages over steroid and metallothionine systems in that it is non toxic to the cell, and basal expression of the controlled gene is virtually eliminated.

**Murine Mast Cell Line System:**

N. Expression of the anti c-*kit* Hammerhead Ribozyme in the P815 Mastocytoma.

P815 cells were co-transfected at a 10:1 ratio with the pCDNA1 vector containing the ribozyme, and the pSSC9 vector which confers neomycin resistance. Vector controls are included. Cells were selected with G418 and expanded in culture. Whole populations were collected and stained with a biotinylated anti murine c-*kit*, followed by a Streptavidin-PE conjugate. Cells were analyzed by Flow Cytometry. Controls included: 1) Mock transfected P815 cells with avidin-PE only (no anti c-*kit* antibody), and 2) Mock transfected P815 cells with biotinylated anti murine c-*kit* antibody plus avidin-PE conjugate. Shown in Figure 15 are the mock transfection control and 3 representative P815 populations which demonstrate repressed Kit protein expression. P815 cells with repressed Kit expression could not be maintained in culture.

O. The Tetracycline Inducible Ribozyme:

We showed that P815 cells transfected with the anti-murine c-*kit* hammerhead ribozyme showed decreased Kit expression relative to controls. However, we believe that inhibition of the Kit RTK in this mastocytoma will result in apoptosis or a severe impairment in its ability to proliferate. The Kit receptor tyrosine kinase in the P815 line is known to have an activating mutation in the kinase domain, and it is thought that this is a large reason why P815 cells are a mastocytoma. This hypothesis is consistent with the observation that these $Kit^-$ populations cannot be maintained in culture.

In order to circumvent this problem, we introduced the ribozyme into the tetracycline inducible system. Our objective with this system was to isolate a clone of the P815 line that would express a high level of the ribozyme in the absence of tetracycline. Our approach to isolate such a clone was to transfect a stable cell P815 clone (P815 15-1) that contained the pUHD15-1 plasmid. P815 15-1 was derived by co-transfecting the pUHD15-1 and pSSC9 (which confers G418 resistance) vectors

into the P815 line. Cells were selected using 1mg/ml of G418 to ensure that the resultant clone would possess a high copy number of pUHD15-1. This should ensure high expression of the transactivator protein tTA.

P. Luciferase assay for Tetracycline Inducibility

In order to assess the functionality of the Tetracycline Inducible System in P815 cells, a stable clone of P815 was established with the pUHD15-1 and pSSC9 vectors (P815 15-1 cells). P815 15-1 cells were loaded with tetracycline for 48 hours, and a series of transient transfections were performed using the pUHD13-3 plasmid. pUHD10-3 is a variant of pUHD10-3, except that it has a luciferase reporter gene in the MCS. Cells were cultured overnight, and then assayed for the presence of the luciferase enzyme with the Luciferase Assay Kit (Promega). Light Output was measured using a Luminometer. These results show that tetracycline quite effectively prevents synthesis of the luciferase enzyme, with very little background expression (Figure 15). Furthermore, in the absence of tetracycline, luciferase is quite adequately expressed demonstrating that this system works well in the P815 line.

Q. Inducible Expression of the Anti c-kit Ribozyme in the P815 mastocytoma

The second step was to co-transfect P815 15-1 cells with pUHD10-3(ribozyme) and p3'SS (confers hygromycin resistance). Cells were selected using 1mg/ml of hygromycin, to ensure that the cells possessed high copy number of the ribozyme containing plasmid. Cells were maintained in 1ug/ml of tetracycline at all times in order to prevent unwanted ribozyme expression.

From a total of five experiments to isolate G418 and hygromycin resistant cells, doubly resistant cells were obtained three times. These cells were single cell cloned and either assayed immediately for ribozyme expression or frozen. The assay consisted of splitting a clonal population in two, and washing one half free of tetracycline. These cells would be cultured for 48 hours and then analyzed for inhibition of Kit expression by Flow Cytometry. Several isolates showed variable inhibition of Kit expression upon tetracycline removal (Figure 16). Unfortunately, these results could not be repeated using these clonal populations. Although cells retained drug resistance, the inducibility of the ribozyme in cells maintained in tetracycline could not be repeated after the initial result. Attempts were made to subclone two of the promising populations, #37 and # 39, with no success. Various time assays were also performed to see if Kit inhibition required a longer or shorter culture time.

Our interpretation of these results are that these cells do not maintain the copy number required to express the ribozyme levels needed to block Kit expression in the P815 line. It is unclear if these plasmids are integrated into the genome or retained in an episomal state. If the plasmids are mostly episomal, then it is conceivable that the effective copy number could be reduced during proliferation. These cells divide very quickly; up to 2 to 3 times in a single 24 hour period. This would effectively reduce the copy number 4 to 6 fold with in a 48 hour period.

This would suggest that the problem could be solved by shortening the time between the transfection and the assay. So, we removed the cloning step. Whole populations of P815 cells were transfected with both plasmids of the tetracycline inducible system and pSSC9. These cells were selected with G418 resistance, and assayed for Kit inhibition as stated. This approach worked one out of six tries.

We have also utilized a transient approach. In this model, the tetracycline inducible ribozyme system would be transfected into P815 cells and assayed over a 72 our period post transfection. This time period allows for ribozyme induction after tetracycline removal and recovery from the transfection. The rationale for this approach was that the copy number would be rather high after the transfection, and that the results could be consistently repeated. However, we have found that this approach relies on the ability of the P815 line to be efficiently transfected. In order to separate transfected cells from non transfected cells, we have employed the use of a new system from Invitrogen called Capture Tec. This system involves the use of a plasmid which expresses a single chain antibody on the cell surface. Transfected cells were then be isolated by using hapten coated magnetic beads which recognize the single chain antibody. Although we established a time point, post transfection, that seems to be optimal for expression of this single chain antibody, the capture frequency is low. Co-transfection experiments using either the pCDNA3 (lacZ assay) or the pGreen Lantern plasmid (FITC fluorescence) have confirmed that these cells are difficult to transfect.

In response to this, we have obtained indirectly from the laboratory of Dr. Thierry Boon a P815 cell line clone called P1.htr. This clone was specifically derived for its ability to be efficiently transfected. This hypertransfectability has been confirmed and optimized.

O. In Vitro Cleavage Assays

An additional objective of this project is to demonstrate that the ribozyme has specific catalytic activity against the c-*kit* mRNA. Our strategy for demonstrating this was to perform in-*vitro* cleavage assays using ribozyme RNA and a target RNA carrying the anti-c-*kit* ribozyme hybridization and target site. A specificity control would be provided by using the antisense target RNA. Ribozyme RNA was generated by transcribing a ribozyme template present in the pCDNA1 vector from the T7 promoter. Target RNAs were generated from the pCR2 vector containing RT-PCR amplified P815 sequence containing the hybridization and target GTC. This GTC was shown to actually be a GTT in the P815 line. Sense target RNA was transcribed using the T7 promoter, and antisense target RNA was transcribed using the Sp6 promoter. All RNA transcription reactions were performed using the Ambion transcription kit, and labeled using $^{32}$P -UTP. Plasmid DNA was digested using DNAase. RNAs were purified over a 8% denaturing polyacrylamide gel, and the resulting RNAs were visualized by autoradiography.

Transcription of the ribozyme reveals that the ribozyme does possess catalytic activity as evidenced by its ability to excise excess 5' DNA present in the MCS of the pCDNA1 vector (Figure 17). This ribozyme was designed with a GTC cleavage site upstream of the catalytic portion of the ribozyme. Excess 5' DNA has been shown to be detrimental to ribozyme function. However, transcription of the target template from the pCR2 vector is not efficient using either the T7 or Sp6 promoters. These reactions have been tried on three separate pCR2 clones. We are currently trying to subclone the target RNA into the pGEM4Z vector.

P. Kit Immunofluorescence and the TUNEL Assay

One of the major questions we wanted to address with the tetracycline inducible ribozyme in the P815 mastocytoma was the role of the activated Kit RTK in P815 cell viability. The TUNEL assay was optimized on cells treated with .8uM 5-aza-cytidine over a 20hour period. 5-Aza induces efficient apoptosis of P815 cells. FITC positive nuclei are indicative of apoptotic cells. Non

apoptotic cells display only background FITC.

An additional counter stain to murine Kit was also developed using Quantum Red as the fluorochrome. Eventually, we would want to show that P815 cells containing the active ribozyme have decreased Kit expression. If the hypothesis is correct that these cells undergo apoptosis upon the removal of Kit, then we would see a corresponding increase in FITC labeling in the nuclei.

Q. Western Blot and Immunoprecipitation Data:

An additional application for successful use of the tetracycline inducible ribozyme would be to address Kit mediated signal transduction. We have been interested in the role of Kit and its ability to interact with other immediate early acting signal transducing proteins such as $B_c$ of the IL3/GMCSFR/IL5R complex, Jak1, and Jak2 (29). In preparation for such assays, we have developed Western and Immunoprecipitation assays for each of these proteins.

We obtained two anti-murine Kit polyclonal antibodies from Immunex courtesy of Kim Campbell. One of these antibodies ($P_1$), has been used to detect Kit by western. Immunoprecipitation of Kit has been performed using the ACK-2 antibody. We have shown that 10ug of this antibody can successfully precipitate most of the Kit from a .5mg P815 lysate at 1ug/ul. $B_c$ and Jak2 were immunoprecipitated using 1:100 dilutions in.5mg of P815 lysate at 1ug/ul as well. All antibody/antigen complexes were precipitated using protein A/G. Western Blots for Kit were performed using a 1:1000 dilution of the $P_1$ antibody. Western Blots for $B_c$ and Jak2 were performed using a 1:1000 dilution of the anti $B_c$ and Jak2 antibodies. A goat anti-rabbit HRP conjugate is used as the secondary. Band visualization was performed using the ECL system (Figure 18).

Although the Jak1 antibody has been used to detect the predicted 110 and 130 kDa forms of Jak1 in the P815 line, the I.P. has not been as successful. We are currently considering a new anti Jak1 antibody from Transduction laboratories.

## The Schwannoma Cell Line System

Recently, we undertook the problem of examining Kit expression in four malignant schwanoma lines, ST88-14, STS26-T, NF1T, and T265-C. We have successfully detected both c-*kit* message and Kit protein in these lines.

R. RT-PCR analysis and Sequencing of schwannoma c-*kit* RNA.

RT-PCR analysis for c-*kit* message was revealed in all four lines using three different primer sets (Figure 19). These individual PCR products were TA cloned and sequenced. The results demonstrated human c-*kit* sequence. We also examined the sequence of the kinase 2 domain (Figure 20) for the presence of the published D-Y mutation associated with activation (30). Although this mutation was not apparent, an additional point mutation seven codons downstream was noted. A preliminary screen has identified this mutation in all four of these lines. This mutation is positioned at base number 2488, changing a T into a C. This is a non conservative base change which encodes a histidine residue, instead of the predicted tyrosine residue (codon # 823).

S. Kit Protein Data.

Kit protein expression was first determined using flow cytometry using the YB5.B8 anti- human Kit antibody (Figure 21). These results demonstrated that only the ST88-14 line had surface expression of the Kit protein. A control was provided by the MO7E line which express high amounts of Kit.

We next tried to detect Kit expression by immunofluorescence. Each Schwann cell line was grown at low density on a cell culture chamber slide, fixed, and then treated with acetone for 15 minutes. Cells were stained with an anti-human Kit monoclonal from Boehringer Mannheim overnight. A FITC conjugated secondary was used to detect the anti Kit antibody. Controls were provided using a non human reactive Isotype Matched Control from Biosource. HMC-1 cells were provided as a positive control. The KEL-FIB fibroblast line was used as a negative control. These results demonstrate that these four lines do express the Kit protein. The fluorescent staining is not clear in the black-and-white images required as per the Army final report instructions, but can be seen in the color originals (Figure 22).

Our next approach was to look at Kit expression by Western Blot. The absence of surface expression of Kit on these lines was curious; we suspected either the protein was truncated or was inappropriately expressed in a cytoplasmic manner. One Western blot using the Boehringer Mannheim antibody revealed that an immunoreactive protein of 90-100 Kda (Figure 23). These results were not duplicated using a polyclonal from Oncogene Science that is directed against the COOH terminus of the human Kit protein (Figure 24).

The protein results indicate that these Schwanomas express an intracellular, truncated form of c-kit that may be associated with the membrane. It is also possible that the YB5.B8 epitope is missing in the NF1T, STS26T, and T265-C lines.

We have worked out an I.P protocol for human Kit using the HMC-1 line. Using this protocol, we should be able to definitively address whether or not the protein that is immunoreactive with the Boehringer antibody is really Kit. Furthurmore, this I.P assay will allow us to address the possibility that this protein is activated.

T. Anti-Human c-*kit* Ribozyme.

We have designed an anti human c-*kit* ribozyme that will be implemented in a manner analogous to the mouse ribozyme. This ribozyme targets a site, starting at base 1604, in the human *c-kit* mRNA that corresponds to the TM domain of the human Kit protein. Initially, we will be using this ribozyme in the MO7E line, which expresses high amounts of the Kit protein, and is known to be transfectable. It is likely that the Schwanoma line of choice in these assays will be the ST88-14 line, because its Kit expression is the highest, and it is easiest to transfect. The ribozyme structure is as follows:

I II I III IV III

attg/gtc/taat/attacga/ctgatgagtccgtgaggacgaaac/caatcag

*ggcggc

I: Flanking arms to GTC target self cleavage site
II: Self Cleavage site
III: Flanking arms to Ribozyme that mediate hybridization and foldup of the ribozyme.

IV: Catalytic core of the ribozyme.

*An additional ribozyme was created where the first six bases of the catalytic core were changed. This ribozyme is inactive, and will serve as a catalytic control.

Subsection summary of results: We have successfully employed the tetracycline inducible system in the murine P815 mastocytoma. This was accomplished by co-transfecting the pUHD15-1 vector and the pSSC9 vector (confers neomycin resistance). Stable pUHD15-1 transfectants were selected with .5mg/ml of G418. Clones were isolated by serial dilution. P815 15-1 cells expressing the functional pUHD15-1 product (tTA) were identified by using a tTA activated luciferase reporter vector called pUHD13-3. This vector was transiently transfected into P815 15-1 cells. Two clones were selected that displayed maximal luciferase activity, in the absence of tetracycline. In the presence of tetracycline, no luciferase activity above background controls could be detected.

This system was then used to control anti *c-kit* ribozyme activity in the P815 15-1 line. An anti *c-kit* ribozyme, designed to target and cleave the GTC triplet at position 1614 in *c-kit* mRNA, was introduced into the tTA activated pUHD10-3 vector. This vector was co-transfected into the P815 15-1 line with the p3'SS vector (confers hygromycin resistance). Cells were cultured in the presence of G418 and hygromycin. Resistant cells were cloned by serial dilution. Cells were maintained in the presence of 1 ug/ml of tetracycline at all times. Anti *c-kit* ribozyme activity was assayed in the following manner: hygromycin resistant P815 15-1 clones were split in two; one half was washed free of tetracycline and the other half maintained in tetracycline for 48 hours. At 48 hours, tetracycline deficient cultures were assayed for inhibition of Kit expression by flow cytometry. One isolated clone displayed the expected inhibition of Kit expression in the absence of tetracycline. This same clone retained normal expression of Kit in the presence of tetracycline. This data has been presented at the Mid Atlantic Immunobiology Conference, May 3,4,5 1996, in Williamsburg, VA. It was also presented in poster format at FASEB, June 1-6, 1996, in New Orleans, LA. We also plan to present this data at the Impacts of Genomics on Immunobiology Conference in Hilton Head, S.C., Nov. 17-20, 1996. We have also begun to prepare a manuscript based on these results.

Pursuant to utilizing this system in the human schwannoma lines, we have also designed and had synthesized an anti-human *c-kit* ribozyme. This ribozyme will target a GTT at position 1604 in the human *c-kit* mRNA. This ribozyme is currently being cloned into the pCDNA1 and pUHD10-3 vectors. Electroporation conditions for the schwannoma lines have also been established.

Overall summary of results: In the present study, we demonstrate Kit expression by additional human malignant schwannoma lines and further examine expression of SCF isoforms and Kit in neurofibromas and in malignant schwannomas. We report that each of the four human malignant schwannoma lines tested using an RNase protection assay (RPA) expresses the exon 6-lacking (membrane-associated) isoform of SCF exclusively, while the majority of human fibroblast sources express mostly the soluble isoform. In addition, analysis of RNA extracted from neurofibroma tissue revealed expression of primarily the soluble isoform of SCF by this mixed population of cells. Conditioned media from the cell lines used were assayed for the presence of soluble SCF by ELISA. The results of these assays confirmed the findings obtained by RPA. We have found that hyperplastic Schwann cells from NF1 related and non-NF1 related neurofibromas do not express

Kit protein, based on immunohistochemistry of neurofibroma lesions. However, Kit expression by malignant schwannoma lines is indicated by immunofluorescent staining and subsequent analysis by confocal fluorescent imaging using the Meridian Ultima confocal interactive laser cytometer. The results suggest that SCF isoform expression is tightly regulated in Schwann cells and that any Kit expression in Schwann cells may be related to a transformed phenotype.

**Materials and General Procedures**

Cell lines

The human fibroblast lines CCD18, HS68 and MRC-9, and the human fibrosarcoma cell line HT1080 were obtained from American Type Culture Collection, Rockville, MD. The four human malignant schwannoma cell lines were generous gifts of Dr. George DeVries, Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia. The ST88-14 line was derived from a peripheral nerve sheath tumor of a patient diagnosed with NF1 (21), and partially described elsewhere (22,23) . Results obtained using this cell line were confirmed using ST88-14 cells kindly provided by Dr. Jonathan Fletcher, Department of Pathology, Brigham and Women's Hospital, Department of Pediatric Oncology, Dana-Farber Cancer Institute, Division of Hematology-Oncology, the Children's Hospital, and Departments of Pathology and Pediatrics, Harvard Medical School, Boston, MA. Other malignant schwannoma cell lines include the STS-26T line, which was derived from an isolated grade III malignant schwannoma in a patient who did not have NF1 (24), the NF-1T cell line, derived from a malignant soft tissue sarcoma in an NF1 patient (24), and the T265-2c cell line, which was established in the laboratory of Dr. George DeVries from a malignant schwannoma in a patient with NF1. The HMC-1 cell line was derived from a patient with mast cell leukemia, and was the kind gift of Dr. Joseph H. Butterfield, M.D., Allergic Diseases and Internal Medicine, Mayo Clinic, Rochester, MN. All cells except HMC-1 were maintained in Dulbecco's modified Eagle's medium supplemented with 2mM L-glutamine, 50μg/ml gentamicin, 100U/ml penicillin, 100μg/ml streptomycin, and 10% fetal calf serum (cDMEM) in a humidified chamber at 37°C and 5% $CO_2$. HMC-1 cells were grown in Iscove's modified Dulbecco's medium with 10% defined, iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, Utah), 1.2mM monothioglycerol (Sigma Chemical Company, St. Louis, MO), 2mM L-glutamine, 50μg/ml gentamicin, 100U/ml penicillin, and 100μg/ml streptomycin at 37°C and 5% $CO_2$.

Antibodies

An affinity-purified rabbit IgG polyclonal anti-human c-*kit* antibody raised against a synthetic peptide (American Research Products, Inc., Belmont, MA) was the primary antibody used for immunohistochemistry. Control experiments were performed using rabbit IgG purified from serum of non-immunized animals (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Immunofluorescent staining was done with a monoclonal anti-human c-*kit* antibody (clone 1.D9.3D6, Boehringer Mannheim Biochemica, Indianapolis, IN). Non-specific interactions were blocked with a solution containing purified human IgG (Jackson). A mouse $IgG_1$ (Biosource International, Camarillo, CA) was used as a negative control. In the immunofluorescence studies, anti-c-*kit* antibodies were localized using a biotin-conjugated $F(ab')_2$ goat anti-mouse IgG (Jackson),

followed by fluorescein isothiocyanate (FITC)-labeled streptavidin (Pierce, Rockford, IL). Biotinylated anti-mouse Kit antibody (Clone 3CD) was obtained from Pharmingen. ACK-2 was obtained from Gibco-BRL. Polyclonal anti-Kit $P_1$ was kindly provided by Dr. K. Campbell at Immunex. Avidin-FITC, Protein A/G, and ECL reagents were obtained from Pierce.

Plasmids

The following plasmids were used in the anti-Kit ribozyme experiments:
1) pUHD15-1: One half of the Tetracycline Inducible System. This plasmid produces the Tetracycline Binding Protein tTA.
2) pUHD10-3: The second plasmid in the Tetracycline Inducible System. This plasmid contains the Multi-Cloning Site just downstream of the tTA binding promoter sequence.
3) pUHD10-3 1609A: The pUHD10-3 vector with the anti-mouse c-*kit* ribozyme.
4) pCDNA1: A eukaryotic expression vector which contains a normal CMV promoter.
5) pCDNA1 1609B: The pUHD10-3 vector with the anti-mouse c-*kit* ribozyme.
6) pSSC9: A plasmid which confers G418 resistance.
7) p3'SS: A plasmid which confers Hygromycin resistance.
8) pCEP4: A Eukaryotic Expression Vector; this vector contains a hygromycin resistance cassette.
9) pCEP4KIT: pCEP4 with the full length human c-kit cDNA.
10) pCEP4DN: pCEP4 with the full length human c-kit cDNA minus the kinase cassette.

The Tetracycline Inducible System (pUHD10-3, pUHD15-1, pUHD13-3) was obtained from Dr. K. Valerie by permission from H. Bujard. pGKneo was obtained from Dr. Micheal Gottesman. pCDNA1, pCDNA3his/lac, and pHook vector systems were obtained from Invitrogen. pGEM4Z was obtained from Promega.

ELISA for Human SCF

Conditioned media were assayed for immunoreactive stem cell factor using a Quantikine™ human SCF kit (R&D Systems, Minneapolis, MN). The kit contains all the necessary antibodies and standards, and the manufacturer's suggested protocol was followed. The plates were read at 450 nm on a V-max Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, CA). The range of the assay was from 31.25 to 1000 pg/ml.

RNase Protection Assay

Total RNA was obtained by using the Ultraspec™ RNA isolation system (Biotecx Laboratories, Inc., Houston, TX). Samples were stored at -80°C at concentrations of 1-2 µg/µl until needed. The probe was generated by PCR amplification of a fragment of the cloned human stem cell factor cDNA including most of exons 5 and 6. The following primers were used: Primer 1: 5'-ATG**GGATCC**ATTCAAGAGCCCAGAAC-3'; primer 2: 5'-GC**TCTAGA**TGCT ACTGCTGTCATTCC-3'. Nucleotides in bold type represent restriction sites for BamHI and XbaI, respectively. After amplification, the fragment was digested with the two enzymes and ligated into pBluescript II SK+ (Stratagene Cloning Systems, La Jolla, CA), which had been similarly digested. A radiolabeled transcript was obtained with the MAXIscript™ In Vitro Transcription Kit (Ambion,

Inc., Austin, TX) using T3 RNA polymerase and ($\alpha$-$^{32}$P) UTP. RNase Protection Assays (RPA) were performed with the RPA II™ kit (Ambion), following the suggested protocol. Fragments were resolved on a 5% acrylamide 8M urea sequencing gel. Protected fragments of 222 and 144 nucleotides are indicative of the exon 6-containing (soluble) and exon 6-lacking (membrane-bound) isoforms of human SCF mRNA, respectively.

Immunohistochemistry

Immunohistochemical staining for human Kit was performed using standard techniques. Briefly, slides containing formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylenes and rehydrated in a series of graded ethanol washes. Endogenous peroxidase activity was blocked with 0.3% $H_2O_2$ in methanol. A polyclonal rabbit anti-Kit antibody was used as the primary antibody at a concentration of 0.5μg/ml. This antibody was then localized using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine (Vector Laboratories) was used as the color substrate.

Immunofluorescence

Each of four human malignant schwannoma cell lines, and the KEL-FIB human fibroblast line (negative control) were cultured at low density on 2-well chamber slides (Nunc Inc., Naperville, IL) in cDMEM until firmly attached. The non-adherent HMC-1 cell line (positive control) were grown in a tissue culture flask and then transferred to slides using a Cytospin 2 (Shandon, Inc., Pittsburgh, PA). All specimens were fixed by immersion in acetone for 15 minutes at -20°C, drained, rinsed in deionized water and dried. 500μl of blocking solution (1% BSA, 100mM Tris pH 7.4, 150mM NaCl) containing 10μg/ml human IgG were added to each chamber, and slides were incubated at 4°C for 30 minutes. The blocking solution was removed and replaced with blocking solution containing either 20μg/ml anti-human c-*kit* or $IgG_1$ isotype control antibodies, followed by incubation at 4°C for 20 hours. Slides were washed 3 times in wash solution (100mM Tris pH 7.4, 150mM NaCl) for 10 minutes each. 0.5ml of a 1:500 dilution of a biotinylated $F(ab')_2$ goat anti-mouse IgG in blocking solution was added to each slide for 1 hour at 4°C. Slides were again washed three times in washing solution, and then incubated in 0.5ml of blocking solution containing a 1:1000 dilution of streptavidin-FITC at 4°C in the dark. Coverslips were placed on the slides using 5% N-propyl gallate (Sigma Chemical Company, St. Louis, MO) to prevent quenching. Coverslips were sealed with fingernail polish. Slides were analyzed on the Meridian Ultima confocal interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI).

RT-PCR and Primers

The ribozyme target sequence was generated by using the following primer set (sense: TGAGGATCCTAAAGAGCAAATCCAGGC; antisense: CATGGATCCCCATTTATCT CCTCGACAAC). The primer set used to amplify the c-kit kinase two region containing the D to V point mutation are as follows: (sense: CCAACCAAGACAGACAAG; antisense: CCATA GGACCAGACATCAC) All RT-PCR and PCR reactions were performed on the Perkin Elmer 9600 model. One μg of P815 total RNA was annealed to Oligo dT and heated to 70C for 10 minutes. Samples were ice chilled. The Reverse Transcription reaction was carried out at 42C for 50 minutes,

followed by a five minute incubation at 90C. Samples were cooled for 15 minutes on ice, and RNA/DNA hybrids were digested by the addition of RNAase H for 20 minutes at 37C. Primers were added to the mixture in a total volume of 75ul at .2uMol. PCR was performed at 94C 15 seconds, 55 for 15 seconds, 72C 30 seconds, for 30 cycles, followed by a 72C soak for 2 minutes, followed by a 4C soak. Samples were analyzed by gel and EtBR staining.

The hammerhead ribozyme oligo (GATCCTGTGGTCGCAGCAGCTGCCTGATGAGTCCGTG AGGACGAAACCACAAAGC) and ribozyme null control (GATCCTGTGGTCGCAGCAGGGC GGCGATGAGTCCGTGAGGACGAAACCACAAAGC) were introduced into the pCDNA1 vector MCS at the BamHI and XhoI sites. For in-vitro transcription, vectors were digested with XbaI or HindIII. Ribozyme RNA was then directionally transcribed from the T7 or Sp6 promoters using the Ambion In Vitro Transcription Kit. Radioactive labeling of the ribozyme RNA was achieved using $P^{32}$ UTP. RNA products were loaded directly onto a 8M urea, 8% polyacrylamide gel and separated using gel electrophoresis. The gel was wrapped into saran wrap and exposed directly to film.

In Vitro *Trans* Cleavage Reaction

The Ribozyme target sequence in the P815 line was amplified by RT-PCR and TA cloned into pCR2.0 (Invitrogen). This sequence was sub-cloned into the pGEM4Z vector at the EcoRI site. Ribozyme target sequence and antisense control were transcribed from the T7 and Sp6 promoters, respectively. Radioactive labelling of the target RNAs were achieved using $P^{32}$UTP. RNA products were loaded directly onto an 8M urea, 5% polacrylamide gel and separated using gel electrophoresis. Gel bands were located by autoradiography and excised. RNA was eluted overnight in RNA elution buffer (1% SDS, 5M $NaOA_c$ in DEPC treated Milli-Q.) Ribozyme RNAs were added to target RNAs in the indicated ratios in a total volume of 2ul, heated to 80C for 5 minutes, then ice chilled. The cleavage reactions were initiated by adding 1ul of ribozyme salt solution (150mM $NH_4SO_4$, 30mM $MgCl_2$, and 10mM Tris pH=7.2 ) and heated for one hour at either 37C or 55C. Reaction was stopped by adding loading buffer. Samples were heated to 70C, and loaded directly onto a 55C sequencing gels. Products were resolved and visualized by autoradiography.

Establishment of the P815 15-1 line:

Cells of the P815 highly transfectable subclone (P1.HTR) were electroporated with 20ug of pUHD15-1 and 2ug of pGKneo at 250uF, 400V one time, and allowed to recover over a 48 hour period. G418 in cDMEM was added at .5mg/ml. Cells were cultured until a mock transfected control cultured in G418 had no viable cells. Clonal cells were picked and expanded. P815 cells expressing a functional pUHD15-1 product, the transactivator tTA, were identified using the pUHD13-3 vector in a transient transfection assay. pUHD13-3 contains a luciferase reporter gene. Clones expressing high luciferase activity in the absece of tetracycline, but only background levels of luciferase in the presence of tetracycline were used in this report.

Transient Transfection Assays

P815 15-1 cells were cultured in tetracycline for 48 hours prior to transfection. The cells were

transfected with a 2:1 ratio of 50ug of pUHD10-3(1609A) and the pHook vector. Transfection efficiency was assessed with a 1:1 pHook and pCDNA3his/lac transfection control. Cells were plated for 24 hours and treated with .25% Glutaraldehyde, washed in HBSS, and incubated in a developing solution containing 2% X-GAL, 4mM $K_3Fe(CN_6)$, 4mM $K_4Fe(CN_6)$-$3H_2O$, 2M $MgCl_2$ in HBSS, pH=7.4. Cells were incubated at 37C for 12 hours. Blue cells were enumerated per five fields and expressed as a percentage of the total cell number counted.

Flow Cytometry

Aliquots of cell cultures were treated with Ficoll Hypaque for 30 minutes to remove cell debris. Cells were washed in cDMEM, pelleted, and resuspended in 100ul of cDMEM. Cells were maintained at 4 C at all times. 4 ug of biotinylated anti kit antibody was added for 30 minutes. Cells were washed with 1ml of cDMEM, and pelleted at 1200 RPM for 10 minutes. Cells were resuspended in 100ul of cDMEM. Avidin-FITC was added at a 1 to 1000 dilution for 30 minutes in the dark. Cells were washed in 1 ml of cRPMI without Phenol Red and pelleted at 1200 RPM for 10 minutes. Cells were resuspended in .5ml of cRPMI without Phenol Red and placed on ice. Flow Cytometry was carried out using a Becton Dickinson Flow Cytometer. Dead cells were gated using Propidium Iodide.

Immunoprecipitation and Western Blots

P815 cells were lysed for 40 minutes on ice using the following lysis buffer: 150mM NaCl, 50mM Hepes pH=7.2, 10% glycerol, 1.2mM MgCl, .5% NP40, 10mM EDTA, .5% aprotinin, 1mM PMSF, 1mM $NaVO_4$, and 50ug/ml Leupeptin. Samples were clarified at 14000 RPM for 15 minutes at 4 C. Supernatants were removed and quantified. 500 ug of total protein was added to I.P. buffer (.1% NP40, 150mM NaCl, 50mM Hepes pH=7.2, 10% glycerol, 1.2mM MgCl, 10mMEDTA, .5% Aprotinin, 1mM PMSF, 1mM $NaVO_4$, and 50ug/ml Leupeptin) for a 1ug/ul total solution. 10ug of ACK-2 was used to immunoprecipitate Kit, for 18 hours at 4 C with gentle rocking. Kit complexes were collected by adding 50ul of a 50% slurry of Protein A/G for 2 hours at 4 C with rocking. Protein A/G was pelleted for 15 seconds and washed 2 times with I.P. buffer. Protein A/G was then boiled in 10X sample buffer for 10 minutes. Samples were pelleted at 14000 RPM for 15 minutes. Supernatants were directly loaded onto an 8% SDS-PAGE gel. Proteins were transferred to Immobilon for 20 hours at 150mAMPs in Transfer Buffer (20% MeOH, 25mM Tris, 192mM Glycine). Protein transfer efficiency was judged by the use of Rainbow standards and also by staining the gel with Coomassie. Western blot for Kit was performed by blocking the membrane with blocking buffer (1% Non Fat Dry Milk (NFDM) in TBS; 10mM Tris, pH=7.2, 150mM NaCl) for 30 minutes at 37 C with rocking. Polyclonal anti-kit antibody was added to the membrane in fresh blocking buffer for 20 hours at 4 C, with rocking. Membranes were washed 3X 10 minutes in TBS. A horseradish peroxidase goat anti-rabbit conjugate (Amersham) was used as the secondary, at a 1:1000 dilution in blocking buffer for one hour at room temperature with rocking. Membranes were washed 3X in TBS. Proteins were visualized using ECL, according to the manufacturer's instructions.

TUNEL Assay for Apoptosis

Transfected P815 15-1 cells and controls were cultured for the indicated times. Cells were fixed using 4% Paraformaldehyde in PBS for 15 minutes and washed. Cells were then treated with acetone at -20C for 15 minutes. Cells were briefly washed to remove residual acetone. TUNEL assay was performed by adding 1ul TdT, 2ul FITC-ddNTP, 2ul of TdT buffer, etc. for 1 hour at 37C. Cells were then washed, and visualized in a fluorescence microscope.

**Conclusions**:

The tumors associated with NF1 can be disfiguring, painful and sometimes life-threatening. Since the majority of the cells in these lesions are Schwann cells, it would appear that they arise as a result of a failure of the processes which normally regulate Schwann cell growth. The reason for this failure might seem obvious since neurofibromin, the defective protein responsible for the disease, has been described as a tumor suppressor. However, not all patients bearing the NF1 gene defect develop tumors. This suggests that the faulty regulation of Schwann cell growth is not solely the result of a neurofibromin malfunction, but may require additional anomalies. We have undertaken this study in an effort to better understand the mechanisms which regulate Schwann cell growth. Based upon our previous report that Schwann cells produce stem cell factor, and that the ST88-14 malignant schwannoma cell line simultaneously expresses SCF and its receptor Kit, we investigated the possibility that the growth of NF1-related tumors was due, at least in part, to an SCF/Kit autocrine loop in the Schwann cells. Whereas Kit is readily detectable on the mast cells in neurofibroma tissue, we were unable to detect Kit expression by Schwann cells in neurofibroma tissue by way of immunohistochemistry. **We conclude from these findings that an autocrine growth loop is likely not responsible for the Schwann cell hyperplasia in a neurofibroma.**

Stem cell factor is expressed as either a membrane-bound or soluble protein as a result of an alternative RNA splicing event (15). Although the regulation of isoform expression is not well understood, differential functions of the two forms have been demonstrated. In contrast to the chemotactic activity of soluble SCF for cells bearing the Kit receptor (16, 20), membrane-bound stem cell factor has been implicated as an adhesion molecule through binding to Kit. This type of interaction allows for a more intense and sustained signal transduction process, because intimate contact between the cells is maintained (32). Moreover, accessory membrane proteins are given the opportunity to interact as well, allowing for costimulation of the original signal, or for the initiation of additional pathways. We detected messenger RNA for only the membrane-bound isoform of SCF in each of the four human malignant schwannoma cell lines tested. Although these data are provocative, their significance is still under investigation. Our hypothesis that this preferential isoform expression either contributed to or resulted from the transformed phenotype of these cells has not been supported by further studies in other solid tumors. Only the soluble isoform of SCF mRNA was detected in each of six human small cell lung cancer cell lines and one breast cancer line. More work is needed to determine how SCF isoform expression is regulated, and to better understand the physiologic consequences. **Thus, we have found that these malignant schwannomas may be a good model system in which to study SCF isoform expression,** because most cells which express SCF make both isoforms. The fact that the SCF is membrane-bound does not rule out its ability to signal in an autocrine loop. Indeed, membrane-bound SCF provides a more potent signal that does soluble SCF (15).

Determining which isoform of stem cell factor is expressed by neurofibroma Schwann cells is difficult due to the multicellular nature of the lesion. Fibroblasts, which are a rich source of SCF, represent 10-20% of the cellular composition of a neurofibroma (5). We found that normal skin and keloid-derived fibroblasts exclusively produce the soluble isoform of stem cell factor message . If the fibroblasts within a neurofibroma express SCF mRNA much more abundantly than the Schwann cells, then a total RNA sample extracted from the tissue may contain a disproportionate amount of fibroblast-derived SCF message compared to that produced by Schwann cells. Therefore, the fact that we detected only the soluble isoform in neurofibroma tissue does not rule out the possibility that the membrane-bound isoform may be present in lower quantities. In addition, the cellular origin of the mRNA can not be determined by this method.

We have demonstrated aberrant Kit expression in the ST88-15 malignant schwannoma line (17). Kit expression by the ST88-14 line was confirmed by immunofluorescent staining and confocal analysis, as well as immunoprecipitation with western blotting. However, the precipitated Kit appears to be a truncated form. Also, there appears to be a significant mutation in the kinase 2 casette of Kit, involving a tyrosine to histadine change. The question of Kit expression in the four schwannoma lines (NF1T, ST88-14, STS26T, and T265C) has been extensively addressed. mRNA for *c-kit* has been detected in all four lines using RT-PCR. However, significant surface expression of Kit could not be detected by flow cytometry on any of the lines except the ST88-14 line. **Low level Kit protein expression which could only be detected using an intracellular immunofluorescent protocol on fixed cells may be due to the presence of a truncated form of Kit**, as discussed below.

Western Blot analysis of Kit expression in these lines has shown that the anti-Kit immunoreactive protein is actually 95 Kda, instead of the predicted 120 or 150Kda forms. This truncated form of Kit may be a novel form of Kit that is either secreted or shed from the cell surface. We have developed an ELISA protocol to detect Kit protein in concentrated schwannoma line conditioned medium. We have also begun an extensive characterization of the human *c-kit* mRNA sequence expressed in these cells, by sequencing overlapping TA-Cloned RT-PCR products. This data has already revealed a novel, non conservative point mutation in the kinase two domain that may render the Kit protein constitutively active. Immunoprecipitation and Western Blot protocols for the human Kit protein have been worked out to address the activation state of the Kit protein in these schwannoma lines.

There is significant evidence that stem cell factor and Kit are involved in some malignancies. Coexpression of SCF and Kit has been reported in gynecological tumors, colon tumor cell lines, the majority of surgical breast tumor specimens and tumor cell lines, and at least 70% of small cell lung cancer tumors and tumor-derived cell lines (33-36). Krystal, et al. have shown that transfection of the c-*kit* gene into a small cell lung cancer cell line that only expresses SCF leads to autocrine growth stimulation dependent upon tyrosine kinase activity (37). They also demonstrated that transfection of a kinase-defective form of the c-*kit* gene into a cell line that naturally coexpresses the receptor and ligand resulted in a loss of growth factor independence (37). **Our evidence that Kit is expressed by four human malignant schwannoma cell lines is suggestive that an autocrine loop may contribute to the growth deregulation of these cell lines.**

We are continuing to address these questions by way of recently-established collaborations. After this work was reported in two presentations at the FASEB meeting this past summer in New Orleans, and it was selected for a thirty minute presentation at the Neurofibromatosis meeting this summer in Snowmass, Colorado. At this meeting, we were able to set up the following collaborations with major investigators in the field of neurofibromatosis: Dr. Nancy Rattner has provided us with conditioned media from primary cultures of Schwann cells from wild type and NF-1 knockout mice for use in our mouse mast cell cultures. Conversely, we have agreed to provide her with primary murine mast cells for co-culture on her mouse Schwann cells. Dr. Rattner has also agreed to provide us with frozen neurofibroma tissue for use in *in situ* hybridization experiment for the Kit/SCF complex. Dr. David Viskochil has provided us with paraffin sections of malignant Schwannomas from 5 different patients. This would give us an opportunity to directly examine Kit expression in a panel of malignant schwannoma patients, in addition to the malignant schwannoma cell lines we have already characterized. We will use a non-cost extension to pursue these research opportunities.

**In conclusion, the funded research suggests that the Kit/SCF complex is likely to be important for development of both mast cell and Schwann cell hyperplasias associated with NF-1. Our first publication (17) established a strong foundation on which to address questions of the role of the Kit/SCF complex in Schwann cell and mast cell hyperplasia seen in NF. Clearly, the mast cell hyperplasia observed in NF-1 lesions can be explained by the presence of potent membrane-bound SCF on the many Schwann cells which constitute this lesion. However, Kit is unlikely to play a role in neurofibroma Schwann cell hyperplasia. It is likely to be an important etiologic element in a change from hypocellular hyperplasia (neurofibromas) to hypercellular malignancy (malignant schwannomas), either as a potent autocrine loop or as a mutation in the Kit kinase 2 domain.**

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**Appendix**:

Figure 1 - PCR of mouse and human stem cell factor
Figure 2 - Attempts to RT-PCR hSCF (using various primer sets) and human actin
Figure 3 - PCR of plasmid DNA of hSCF alone or hSCF+102
Figure 4 - RT-PCR of human SCF using a lower primer annealing temperature
Figure 5 - Southern blot of PCR products probing for *neu* differentiation factor
Figure 6 - Scheme for RNAse protection assay for human SCF isoforms
Figure 7 - SCF isoforms in malignant schwannoma cell lines
Figure 8 - SCF isoforms in human fibroblast cell lines
Figure 9 - SCF isoforms in human primary fibroblasts
Figure 10- ELISA for human SCF in malignant schwannoma culture supernatants
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Figure 12 - Anti murine c-kit hammerhead ribozyme
Figure 13 - The tetracycline inducible system
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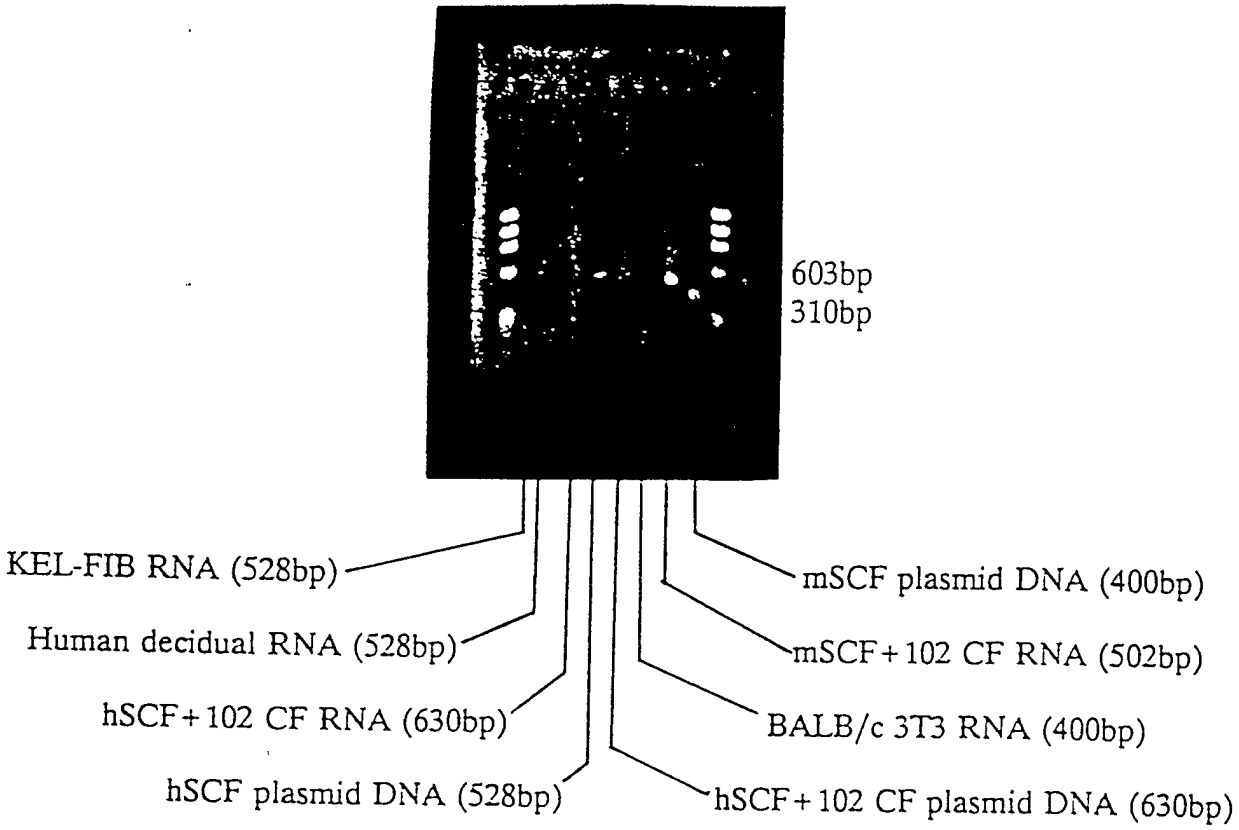
Figure 15 - Luciferase assay for tetracycline inducible system
Figure 16 - Inhibition of Kit expression by a tetracycline-inducible anti-c-kit ribozyme
Figure 17 - Cis in vitro cleavage reaction
Figure 18 - Immunoprecipitation and western blot of Jak2
Figure 19 - RT-PCR analysis of malignant schwannoma Kit expression
Figure 20 - A mutation in Kit of malignant schwannomas
Figure 21 - Flow cytometry analysis of Kit expression on malignant schwannoma lines
Figure 22 - Immunofluorescent staining for Kit expression
Figure 23- Immunoprecipitation with monoclonal antibody of Kit expression in malignant schwannoma lines
Figure 24- Immunoprecipitation with polyclonal antibody of Kit expression in malignant schwannoma lines

Figure 1.

PCR of human and mouse stem cell factor (9-28-93).

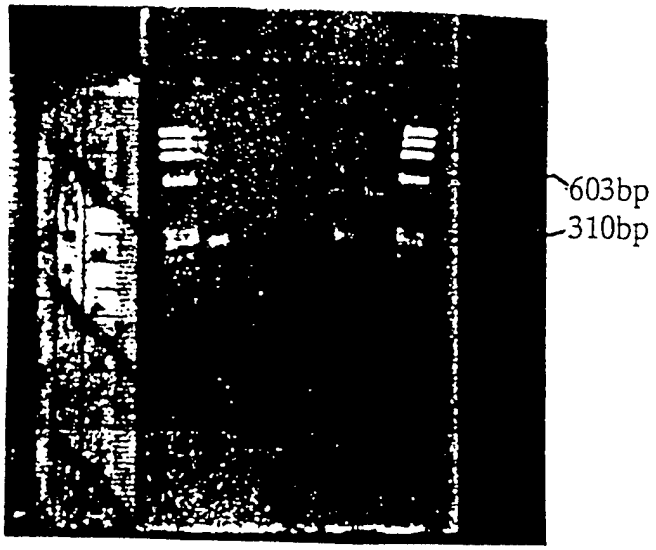



Comments:

None of the human RNA samples were successfully amplified (lanes 2-4).
The mSCF RNA control fragment (lane 8) worked, but the human did not (lane 4).

Figure 2.

Attempts to RT-PCR hSCF (using various primer sets) and human actin (11-18-93).

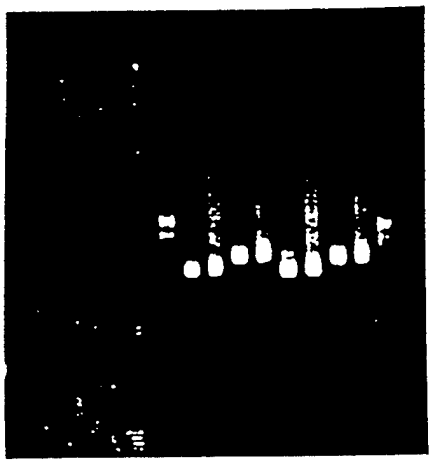



Comments:

Human actin (267bp) was successfully RT-PCR-amplified (lanes 2 and 6), but none of the primer sets were able to amplify human SCF (lanes 3,4,5, and 7).
Note: two different human fibroblast cell lines were used as RNA sources.

Figure 3.

PCR of plasmid DNA of hSCF alone or hSCF+102 (10-14-93).

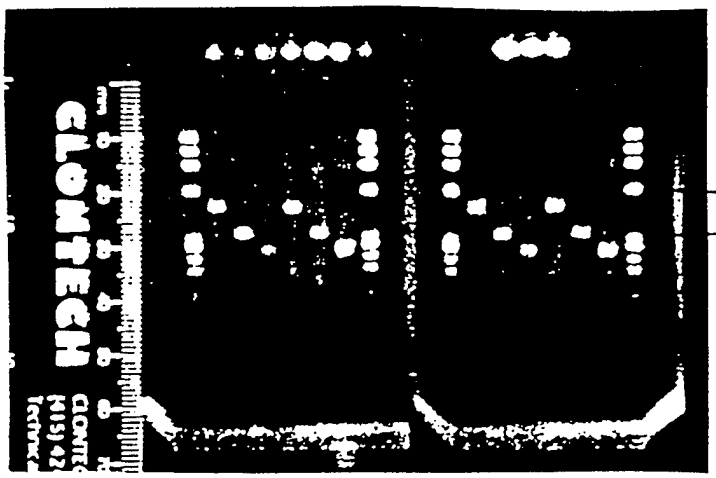


Comments:

Lanes 2 and 3 contain hSCF plasmid and hSCF+102 plasmid, respectively. Lanes 4 and 5 contain the same samples using a different primer set. Lanes 6-9 are simply additional lanes loaded with the same samples as lanes 2-5.
For each pair of lanes, the second sample (hSCF+102) should have migrated more slowly than the first (hSCF with no insert). The 102bp difference in size is not clearly detectable.

Figure 4.

RT-PCR of human SCF using a lower primer annealing temperature (1-31-94).



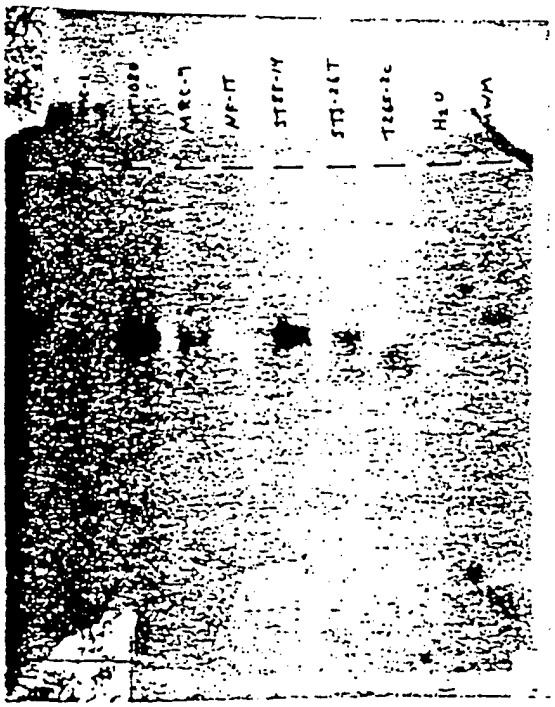

Comments:

Each triplet of lanes represents a different human fibroblast cell line source of RNA. Primers used in each triplet: soluble isoform (481bp), both isoforms (332bp), and human actin (267bp), respectively.

Lowering the primer annealing temperature from 55 to 48 degrees C seems to have greatly improved my ability to RT-PCR hSCF.

Figure 5.

Southern blot of PCR products probing for *neu* differentiation factor (12/28/94).

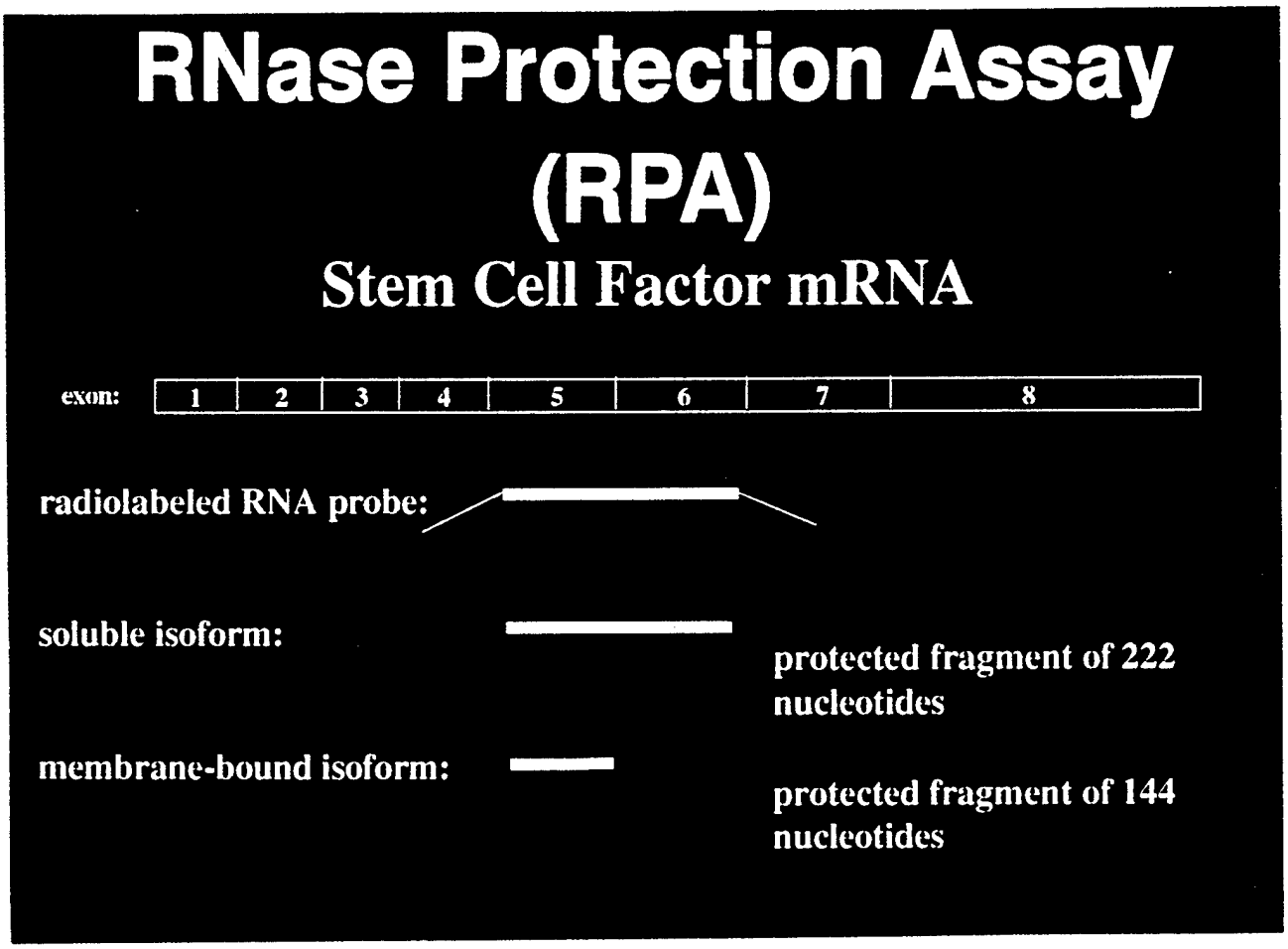


Comments:

mRNA for human NDF is detected in the HT1080 human fibrosarcoma line, the human fibroblast line MRC-9, and the malignant Schwannoma lines ST88-14 and STS-26T. No message is detected in the human mast cell line HMC-1 or in the NF-1T malignant Schwannoma.

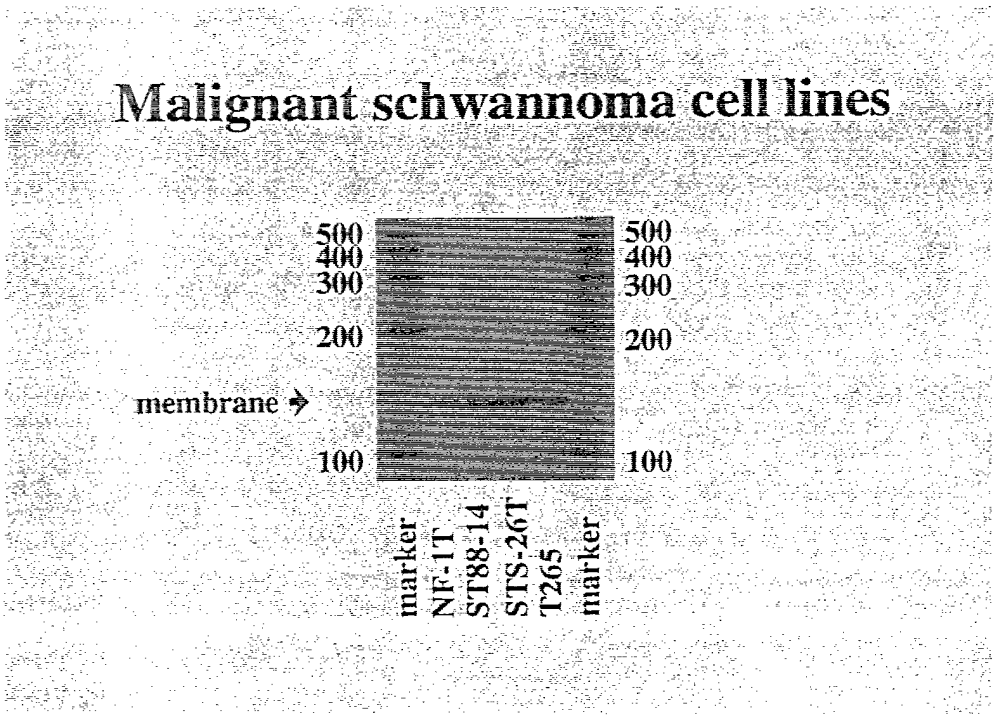
A faster migrating fragment was detected in the T265-2C Schwannoma. This may represent an alternate isoform of NDF.

Figure 6 - Scheme for RNAse protection assay for human SCF isoforms



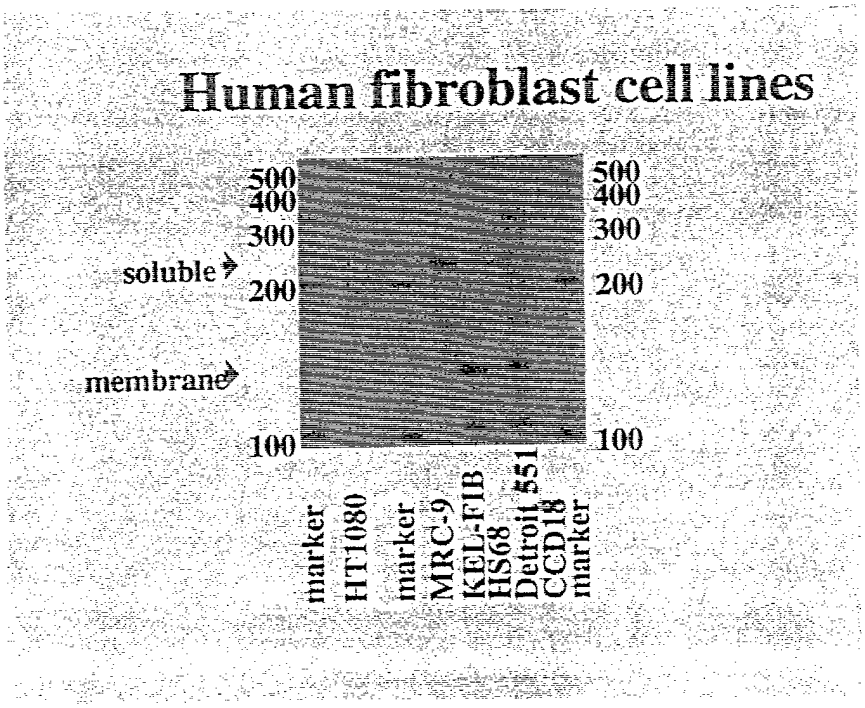

A uniformly radiolabeled RNA transcript containing a sequence complementary to most of exons 5 and 6 of the human stem cell factor mRNA was generated by *in vitro* transcription and allowed to hybridize with 10μg of total RNA. Single stranded RNA was digested with a mixture of RNases A and $T_1$, and the products were resolved by polyacrylamide gel electrophoresis. The presence or absence of exon 6 is indicated by a protected fragment of 222 or 144 nucleotides, respectively.

Figure 7 - SCF isoforms in malignant Schwannoma cell lines



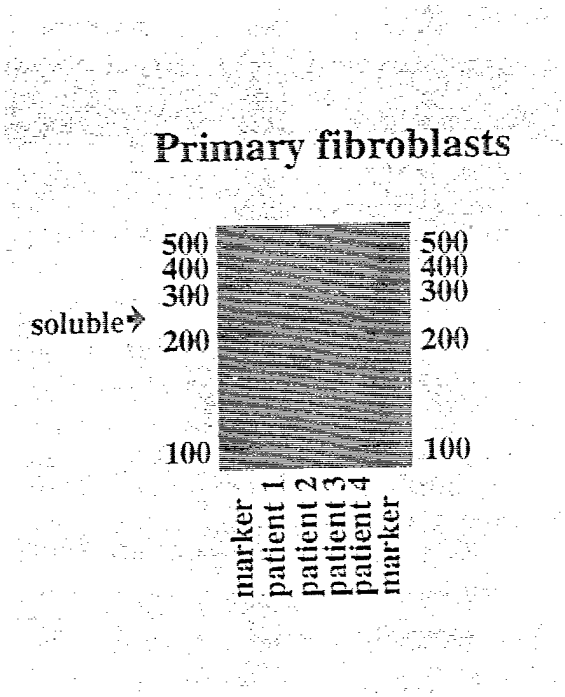

Only the 144 nucleotide protected fragment is observed when each of four human malignant schwannoma cell lines is analyzed, suggesting that these lines express only the membrane-bound isoform of stem cell factor mRNA.

Figure 8 - SCF isoforms in human fibroblast cell lines



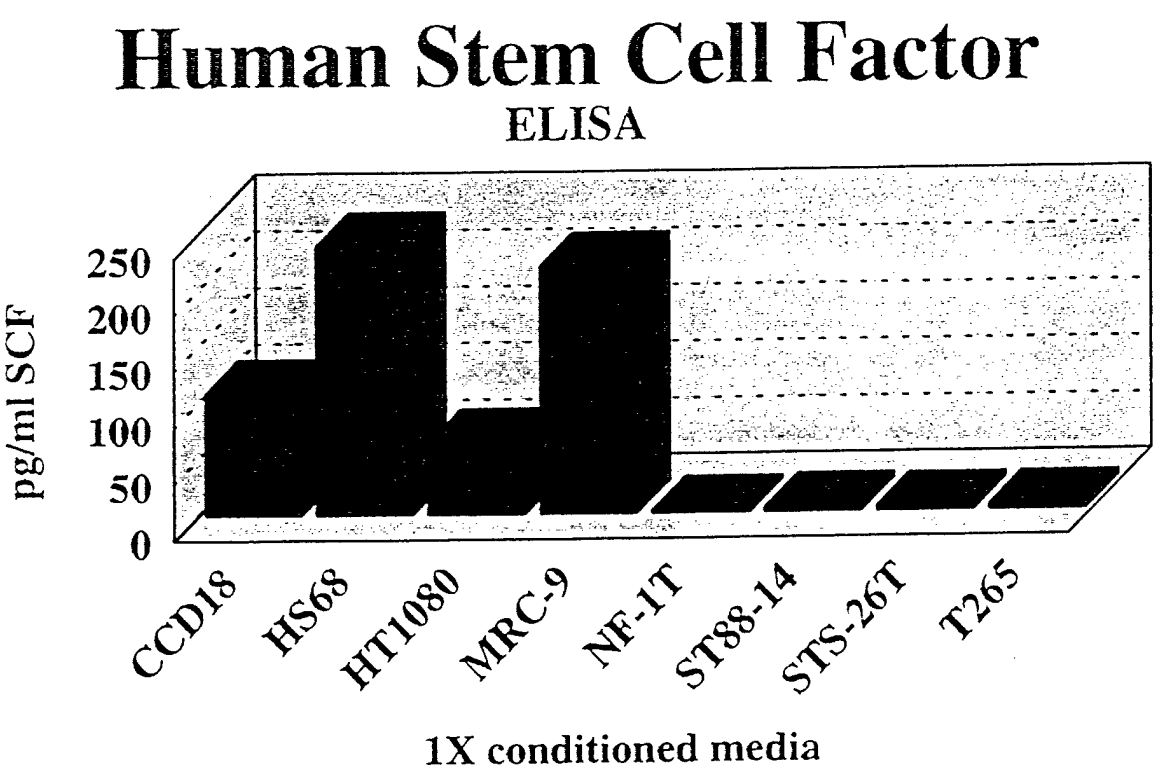

Primary fibroblasts derived by explantation of human skin tissue express the soluble isoform of SCF messenger RNA. Fibroblasts from six additional patients were assayed, yielding similar results.

Figure 9 - SCF isoforms in human primary fibroblasts



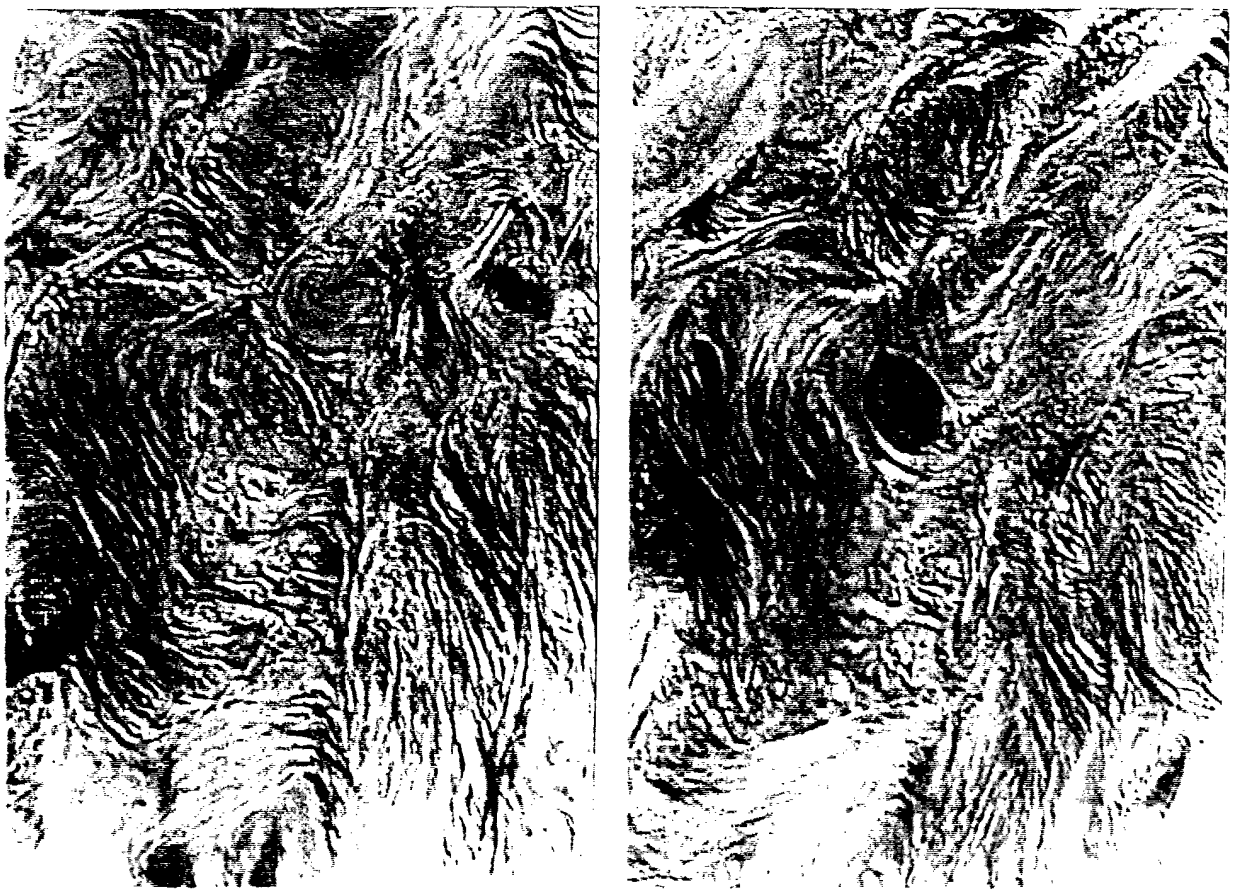

Primary fibroblasts derived by explantation of human skin tissue express the soluble isoform of SCF messenger RNA. Fibroblasts from six additional patients were assayed, yielding similar results.

Figure 10- ELISA for human SCF in malignant Schwannoma culture supernatants



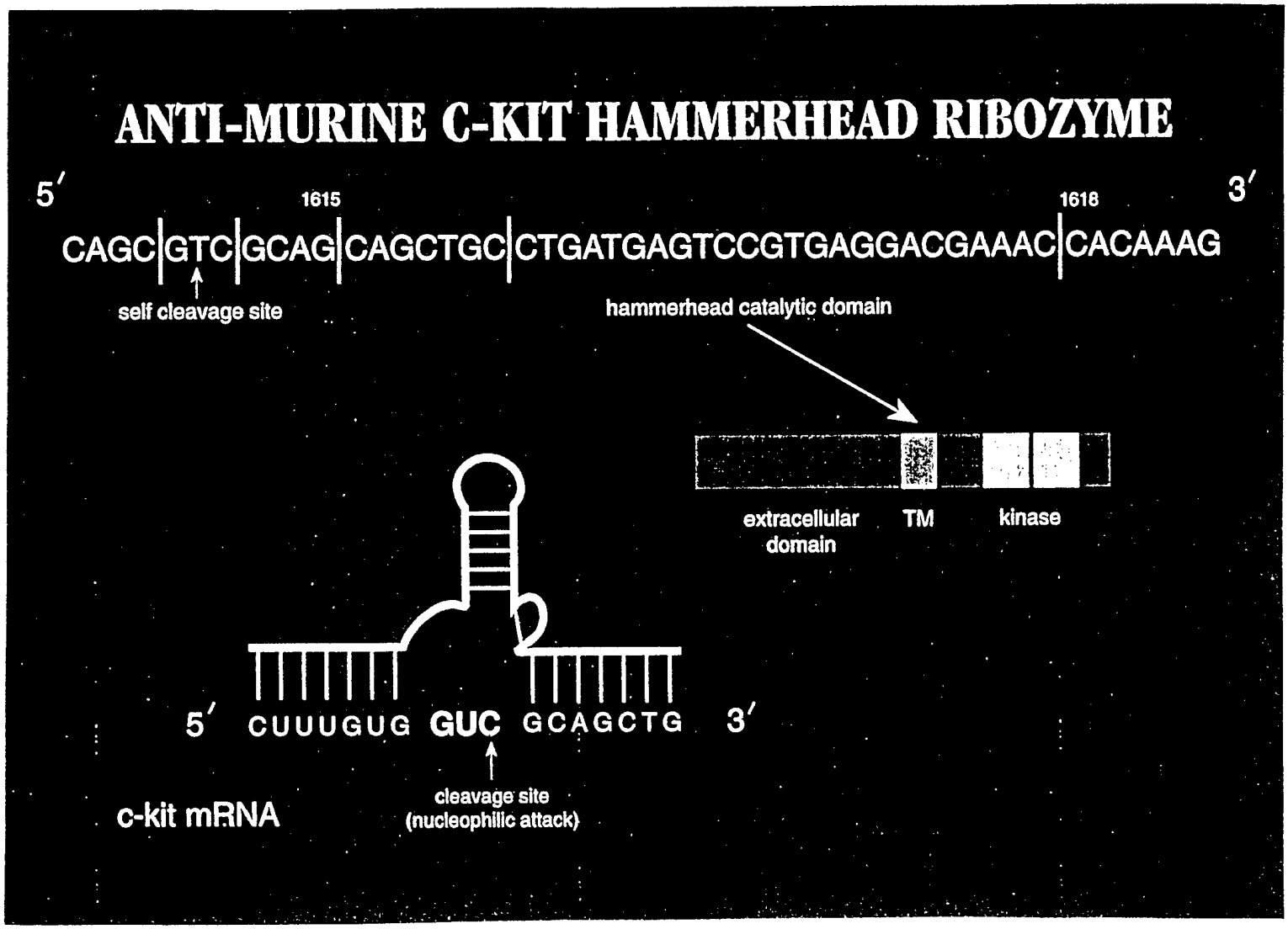

Conditioned media from the cell lines analyzed by RPA were assayed by ELISA for the presence of soluble stem cell factor. The conditioned media of the cell lines used were analyzed for the presence of human stem cell factor using a quantitative sandwich enzyme immunoassay technique. Samples were added to a microtiter plate coated with a monoclonal antibody specific for SCF. After washing away unbound proteins, a horseradish peroxidase-linked polyclonal SCF-specific antibody was added. Following additional washing, a substrate solution containing tetramethylbenzidine was added. Color change was assessed using an automated plate reader, and SCF was quantitated by comparing values obtained with a standard curve. Cell lines for which the soluble isoform was detected by RPA also released SCF into the media, whereas the malignant schwannoma cell lines did not.

Figure 11- Immunohistochemical staining for KIT in NF-1 lesions



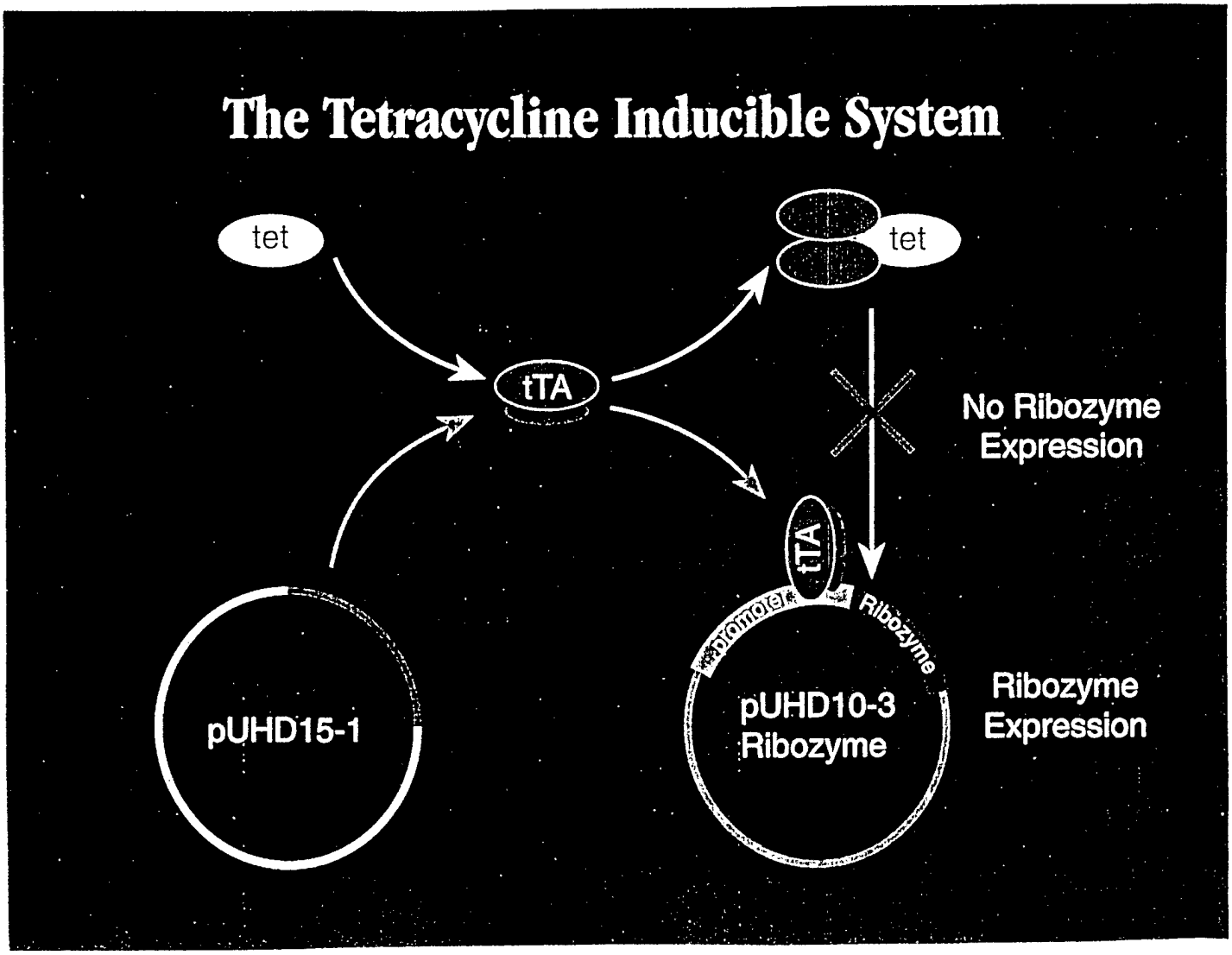
Thin sections (7μm) of formalin-fixed, paraffin-embedded neurofibroma tissue were deparaffinized in xylenes and rehydrated in a series of graded ethanol washes. Endogenous peroxidase activity was blocked with 0.3% $H_2O_2$ in methanol. Specimens were incubated overnight at 4°C in the presence of either an affinity purified rabbit polyclonal anti-human Kit IgG, or an isotype-matched control antibody at a concentration of 0.5μg/ml. The primary antibody was localized by incubating with a biotin-conjugated goat anti-rabbit IgG, followed by avidin complexed with biotinylated horseradish peroxidase. Diaminobenzidine was used as the substrate. Infiltrating Kit-positive mast cells serve as a convenient internal control for this protocol.

Figure 12 - Anti murine c-kit hammerhead ribozyme



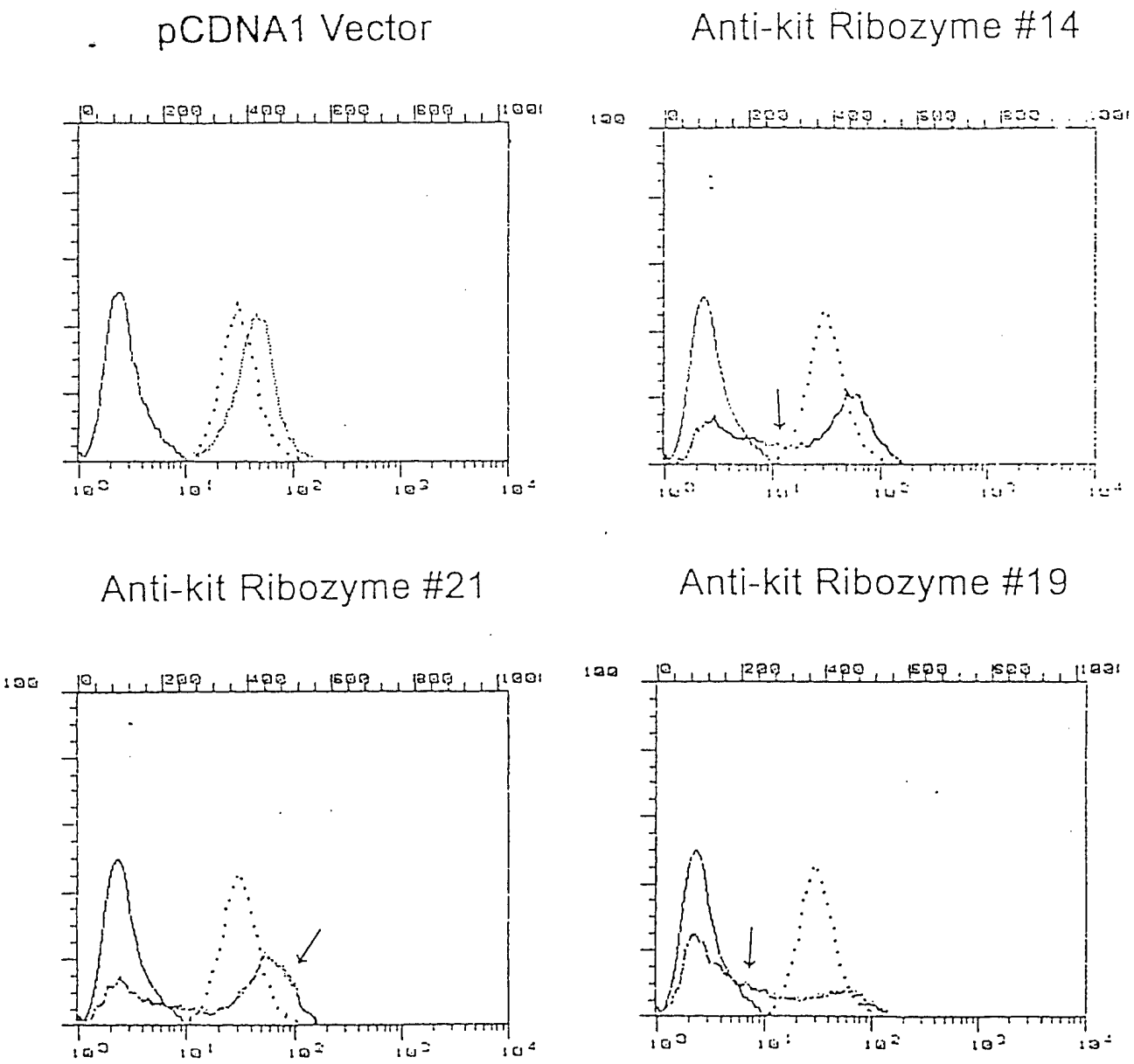

Anti c-kit hammerhead ribozyme design. This hammerhead ribozyme was designed to target a GTC located at bases 1616-1618 based on the published murine c-*kit* sequence. Sequence analysis of the P815 c-*kit* ribozyme target site has shown that this triplet is actually a GTT. This site is located in a region of the c-*kit* mRNA that corresponds to the murine kit protein transmembrane domain. The ribozyme sequence consists of a catalytic core sequence, flanked by two seven base regions that are responsible for mediating hybridization with the flanking sequences surrounding the GTT triplet. Also shown is a self cleavage site located 5' to the catalytic core of the ribozyme. Excess 5' material, often present in multi-cloning sites (MCS), has been shown to be detrimental to ribozyme function. This self cleavage site allows the ribozyme a means to remove this excess 5' material.

Figure 13 - The tetracycline inducible system



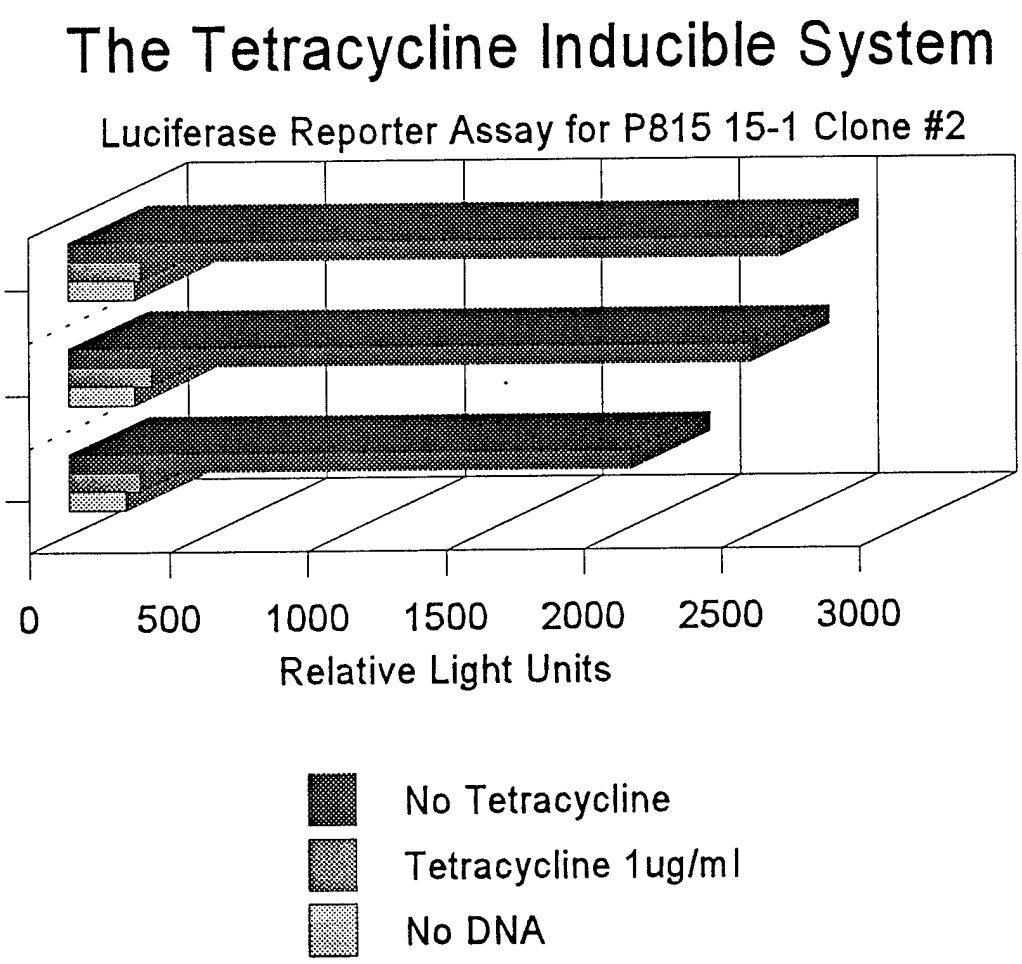

This intracellular expression system consists of two plasmids, pUHD10-3 and pUHD15-1. pUHD15-1 constitutively expresses a tetracycline binding protein called tTA. tTA is a transcriptional activator, which will bind to a promoter sequence located in the pUHD10-3 plasmid. This promoter sequence is located upstream of a MCS which contains the ribozyme. The ribozyme was introduced into this MCS via SacII and XbaI sites (pUHD10-3(1609A)). In the presence of tetracycline, tTA will not bind the promoter sequence, and no transcription of the ribozyme will occur. When tetracycline is removed, tTA binds the promoter and the ribozyme is transcribed.

Figure 14 - Inhibition of Kit expression by an anti-c-kit ribozyme



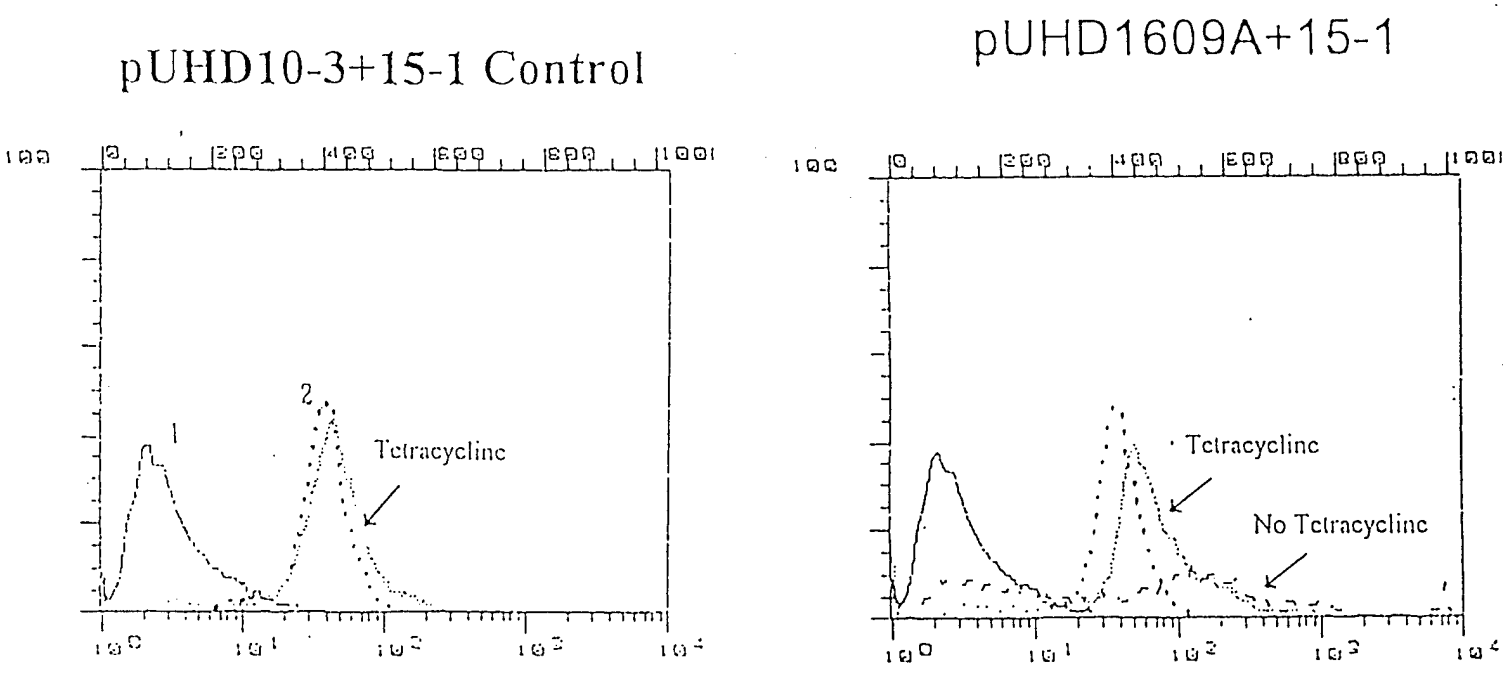

Expression of the anti c-*kit* Hammerhead Ribozyme in the P815 Mastocytoma. P815 cells were co-transfected at a 10:1 ratio with the pCDNA1 vector containing the ribozyme, and the pSSC9 vector which confers neomycin resistance. Vector controls are included. Cells were selected with G418 and expanded in culture. Whole populations were collected and stained with a biotinylated anti murine c-kit, followed by a Streptavidin-PE conjugate. Cells were analyzed by Flow Cytometry. Controls in each figure include: 1) Mock transfected P815 cells with avidin-PE only (no anti c-*kit* antibody), and 2) Mock transfected P815 cells with biotinylated anti murine c-*kit* antibody plus avidin-PE conjugate. Shown are four representative P815 populations which demonstrate repressed kit protein expression. P815 cells with repressed kit expression could not be maintained in culture.

Figure 15 - Luciferase assay for tetracycline inducible system



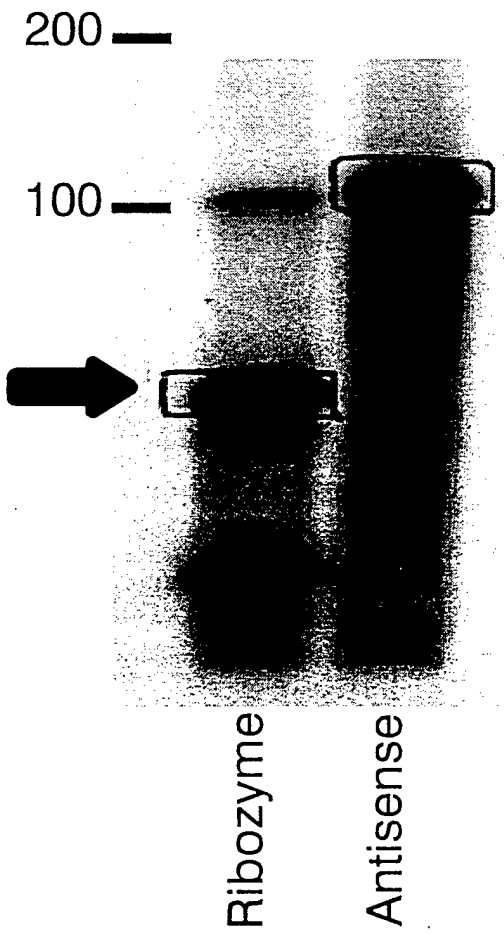

In order to assess the functionality of the Tetracycline Inducible System in P815 cells, a stable clone of P815 was established with the pUHD15-1 and pSSC9 vectors (P815 15-1 cells). P815 15-1 cells were loaded with tetracycline for 48 hours, and a series of transient transfections were performed using the pUHD13-3 plasmid. pUHD10-3 is a variant of pUHD10-3, except that it has a luciferase reporter gene in the MCS. Cells were cultured overnight, and then assayed for the presence of the luciferase enzyme with the Luciferase Assay Kit (Promega). Light Output was measured using a Luminometer. These results show that tetracycline quite effectively prevents synthesis of the luciferase enzyme, with very little background expression. Furthermore, in the absence of tetracycline, luciferase is quite adequately expressed demonstrating that this system works well in the P815 line.

Figure 16 - Inhibition of Kit expression by a tetracycline-inducible anti-c-kit ribozyme




P815 15-1 cells cultured in the presence of tetracycline were co-transfected with the pUHD10-3(1609A) and the pSSC9 plasmid (confers hygromycin resistance) at a 10:1 ratio. Cells were selected for hygromycin resistance, cloned, and expanded in culture. Clonal populations were either assayed immediately or frozen. Clonal populations were split in two. One was retained in tetracycline and the other was washed free of tetracycline. Cells were assayed at 24 hours post tetracycline removal by flow cytometry, using a biotinylated anti-c-kit antibody followed by a Strepavidin-PE conjugate. Controls are as follows; 1) Mock transfectant with avidin-PE only, and 2) Mock transfectant stained with biotinylated anti c-kit plus avidin-PE. Mock transfectants were treated identically to clonal populations excluding drug selection. This population represents the best result of inducible ribozyme mediated kit inhibition in the P815 line.

Figure 17 - Cis in vitro cleavage reaction

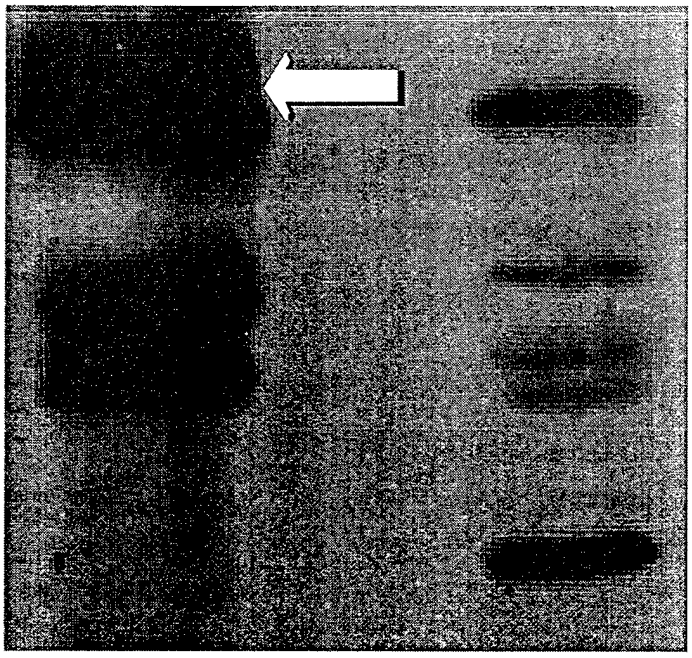



The hammerhead ribozyme sequence was introduced into the pCDNA1 vector MCS at the BamHI and XhoI sites. Vectors were linearized at the XbaI site. Ribozyme RNA was then directionally transcribed from the T7 promoter using the Ambion In Vitro Transcription Kit. Radioactive labeling of the ribozyme RNA was achieved using $P^{32}$ UTP. Irrelevant ribozyme sequence was similarly transcribed and labeled from the opposite direction using the Sp6 promoter. RNA products were loaded directly onto a 8M urea, 8% polyacrylamide gel and separated using gel electrophoresis. The gel was wrapped into saran wrap and exposed directly to film. Shown is a 53 base fragment (arrow) that correctly corresponds to the self cleaved ribozyme. Also present is the cleaved excess 5' material (smaller band). This figure demonstrates that the ribozyme is capable of mediating *cis* cleavage. Irrelevant ribozyme sequence was incapable of mediating this cleavage.

Figure 18 - Immunoprecipitation and western blot of Jak2

# Immunoprecipitation and Western Blot of Jak2.

1 2

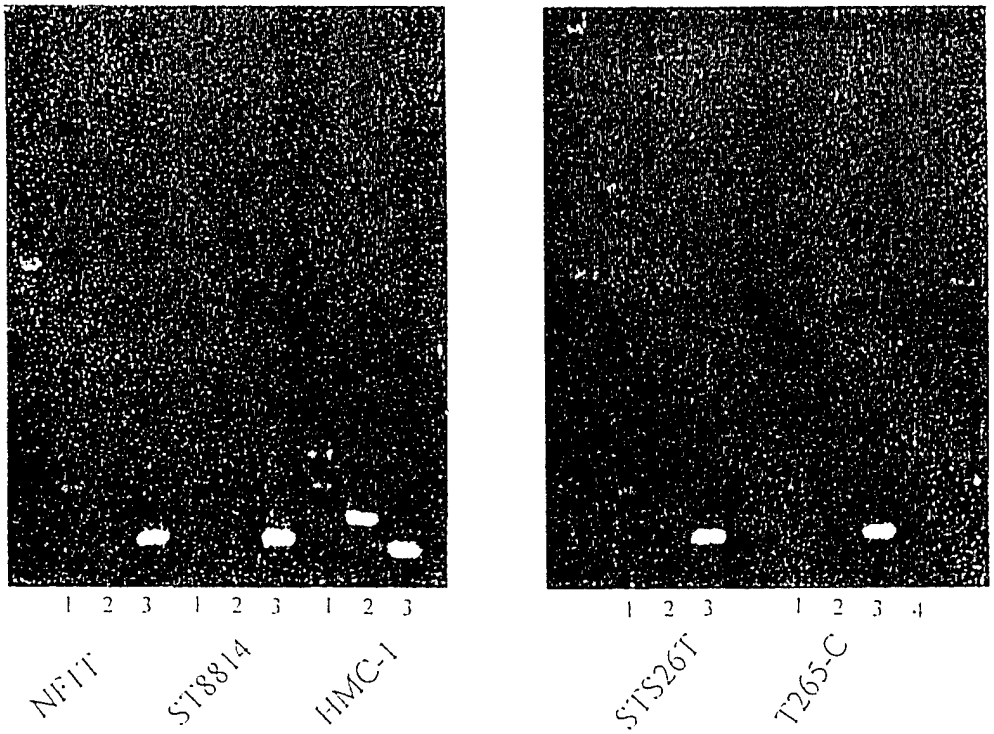


I.P: Anti Jak2 Polyclonal 1:100
Western: Anti Jak2 Polyclonal 1:1000
Lanes:

1. I.P. and Western of Jak2 from 2ug/ul P815 lysates.
2. Original Lysate of P815 2ug/ul.

The presence of Jak2 (indicated by the arrow) was enriched by immunprecipitation with the polyclonal antibody, as indicated by the more intense banding in Lane 1 versus Lane 2.

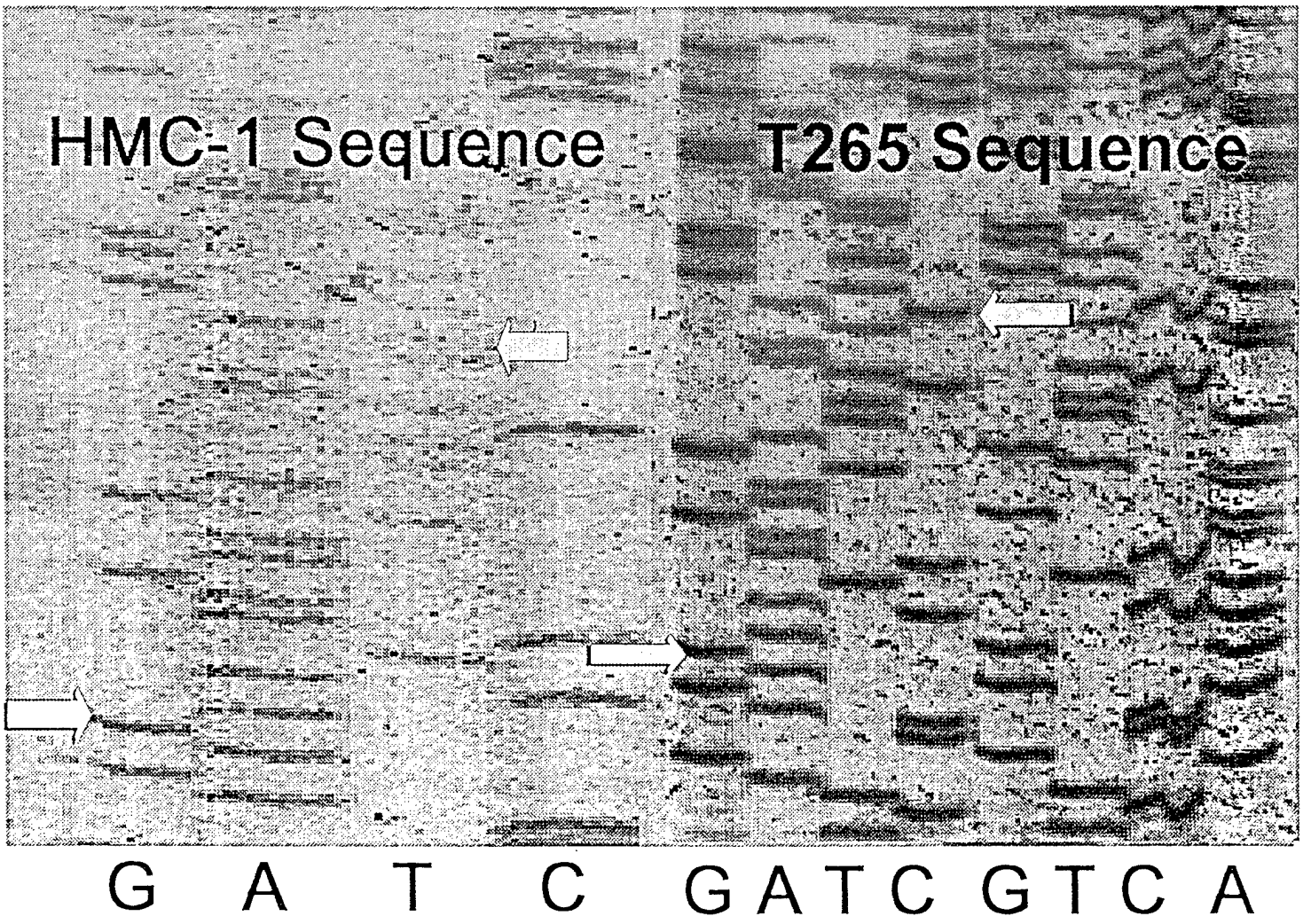
Figure 19 - RT-PCR analysis of malignant Schwannoma Kit expression




Lanes: 1. Kit Primers bases 1259-1691(Extracellular Membrane Proximal and Transmembrane Domain).
2. Kit Primers bases 2262-2587(Kinase #2 Domain).
3. Human Actin.
4. $dH_2O$ Blank

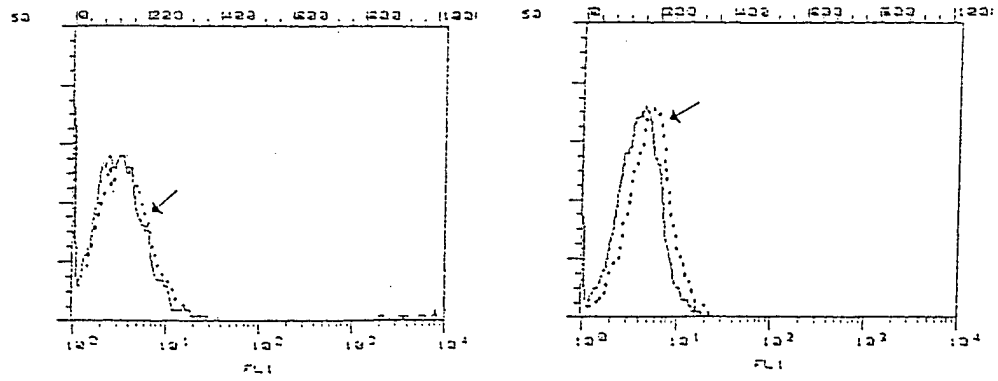
RT-PCR analysis for c-*kit* message was revealed in all four lines using three different primer sets.

Figure 20 - A mutation in Kit of malignant Schwannomas




After RT-PCR analysis for c-*kit* message was revealed in all four lines using three different primer sets, these individual PCR products were TA cloned and sequenced. The results demonstrated human c-*kit* sequence. We also examined the sequence of the kinase 2 domain for the presence of the published D-Y mutation associated with activation. Although this mutation was not apparent, an additional point mutation seven codons downstream was noted. A preliminary screen has identified this mutation in all four of these lines. This mutation is positioned at base number 2488, changing a T into a C. This is a non conservative base change which encodes a histidine residue, instead of the predicted tyrosine residue (codon # 823).

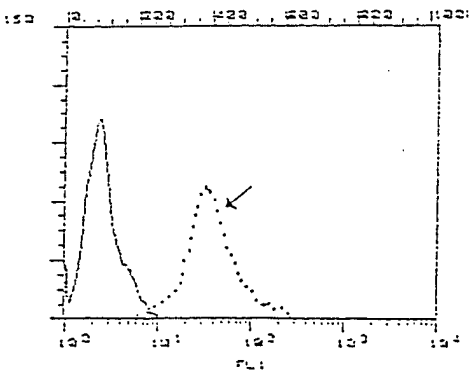
Figure 21 - Flow cytometry analysis of Kit expression on malignant Schwannoma lines



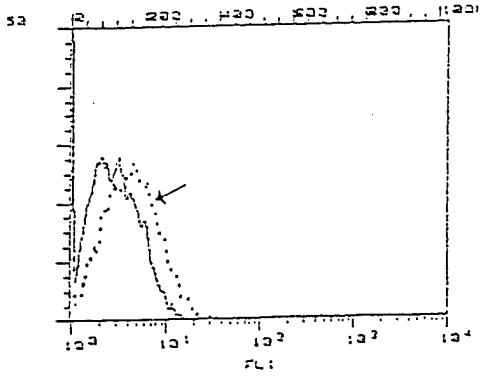
STS-26T



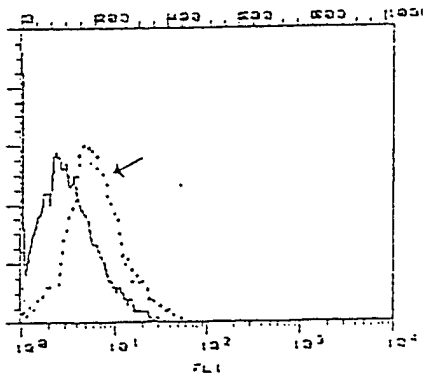
T265-C



MO7E



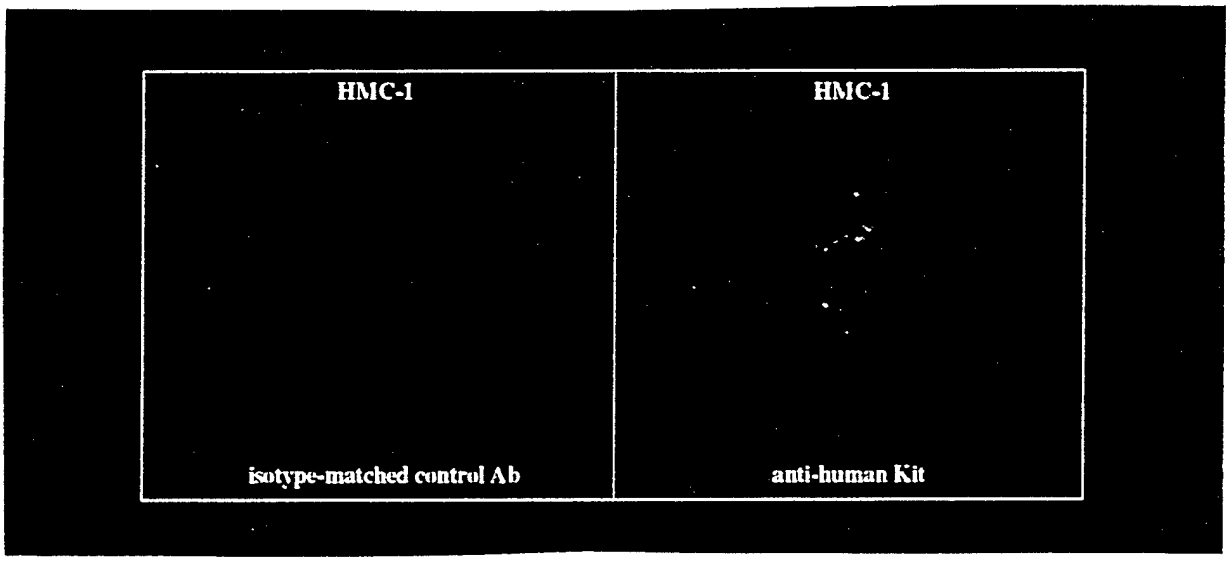
NF1T

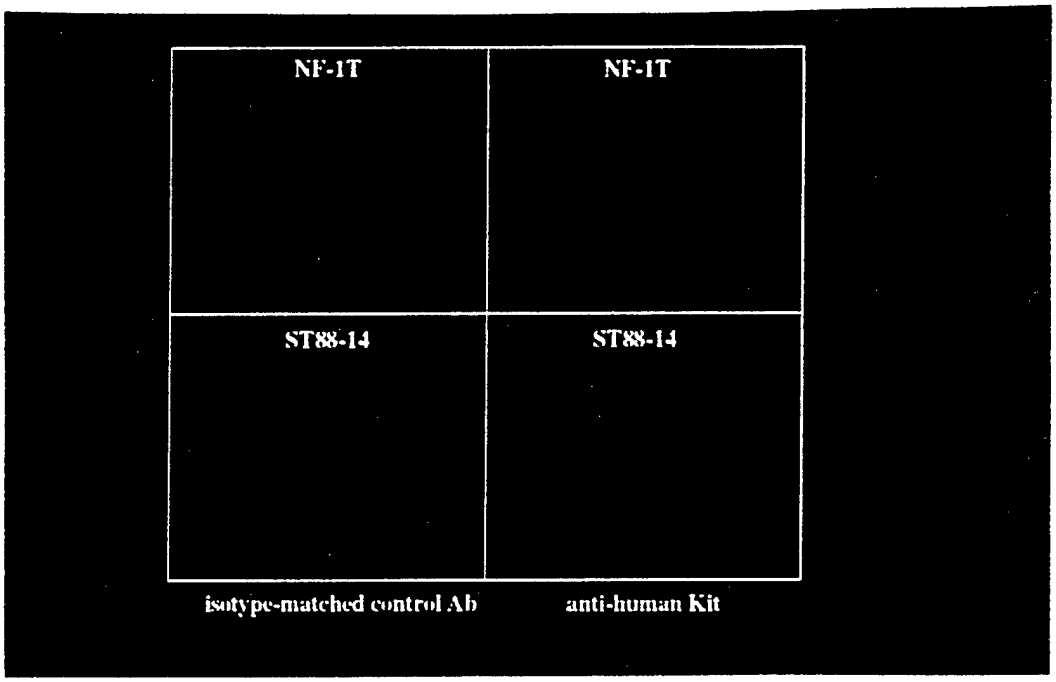


ST88-14

Kit protein expression was first determined using flow cytometry using the YB5.B8 anti- human kit antibody. These results demonstrated that only the ST88-14 line had surface expression of the kit protein. A control was provided by the MO7E line which express high amounts of kit.

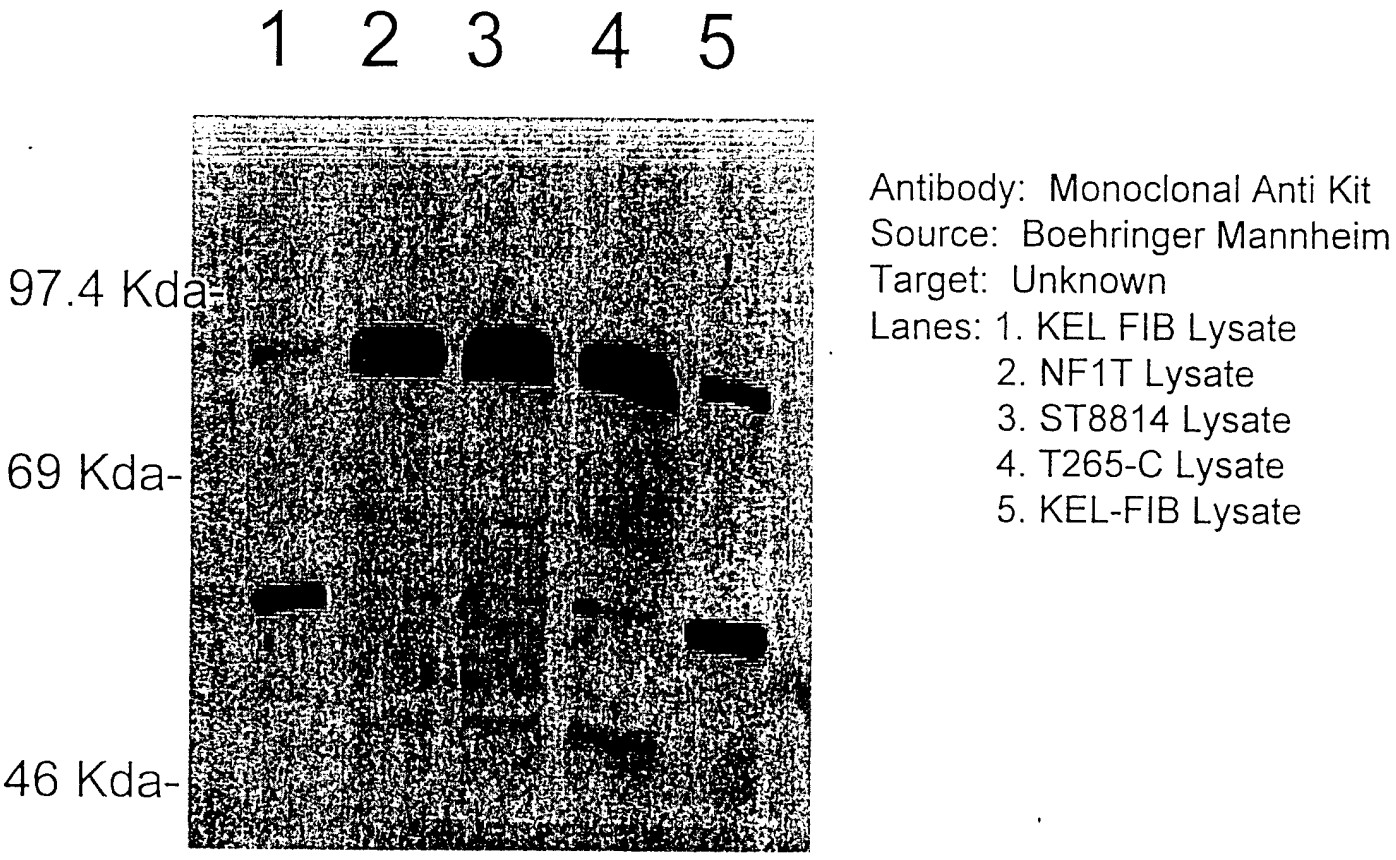
Figure 22 - Immunofluorescent staining for Kit expression



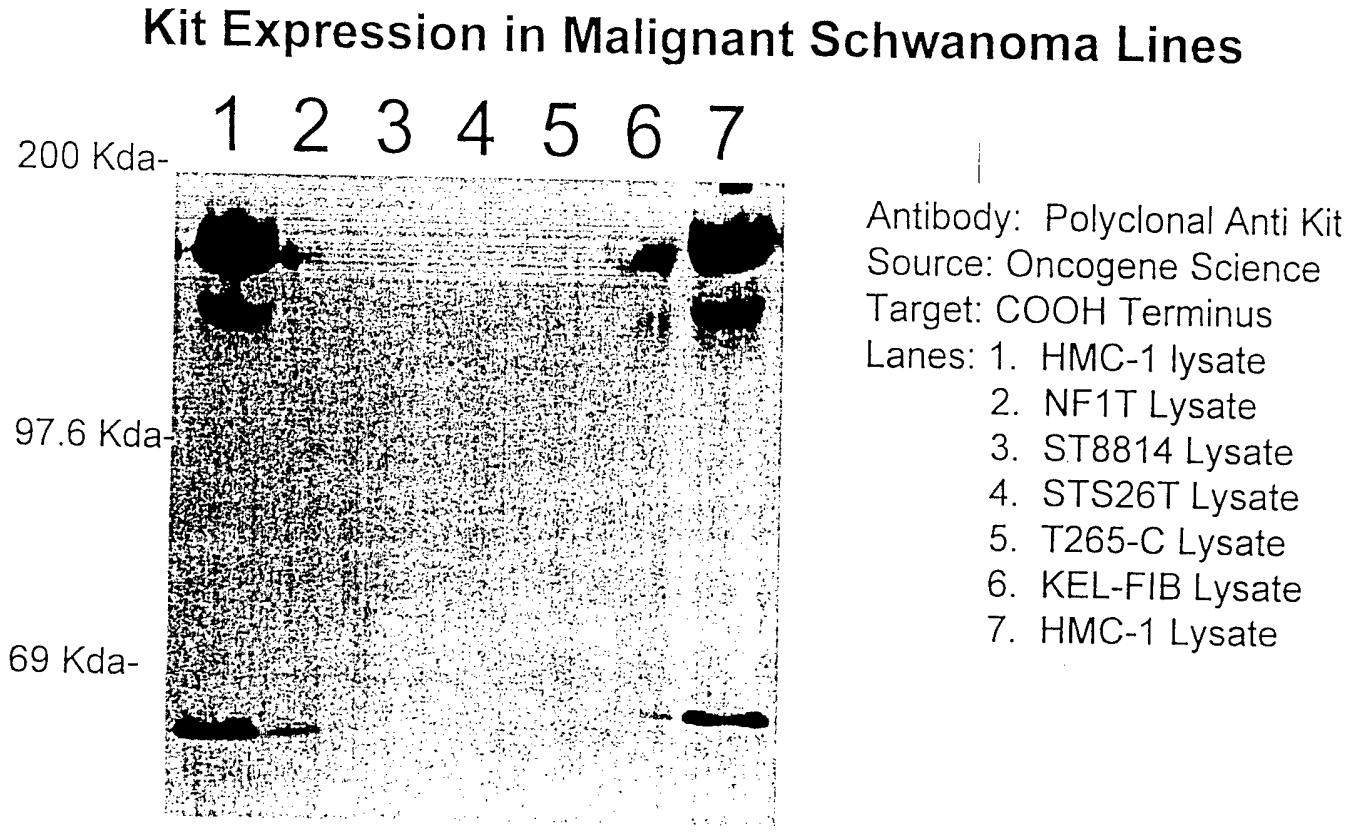

Each Schwann cell line was grown at low density on a cell culture chamber slide, fixed, and then treated with acetone for 15 minutes. Cells were stained with an anti-human kit monoclonal from Boehringer Mannheim overnight. A FITC conjugated secondary was used to detect the anti kit antibody. Controls were provided using a non human reactive Isotype Matched Control from Biosource. HMC-1 cells were provided as a positive control. The KEL-FIB fibroblast line was used as a negative control. These results demonstrate that these four lines do express the kit protein.

Figure 23 - Immunoprecipitation with monoclonal antibody of Kit expression in malignant Schwannoma lines




The western blot using the Boehringer Mannheim monoclonal antibody revealed that an immunoreactive protein of 90-100 Kda.

Figure 24 - Immunoprecipitation with polyclonal antibody of Kit expression in malignant Schwannoma lines




These results from the previous figure (monoclonal antibody) were not duplicated using a polyclonal from Oncogene Science that is directed against the COOH terminus of the human kit protein. The protein results indicate that these Schwanomas express an intracellular, truncated form of c-kit that may be associated with the membrane.

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List of personnel receiving pay from the negotiated effort:
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Karen L. Shanaberger - laboratory specialist A
Kenneth E. Roth - graduate research assistant
Christopher P. Shelburne (AASERT) - graduate research assistant